Freud-1, a Novel Regulator of the Dopamine D2 and Serotonin 1A Receptor Genes

By
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ABSTRACT

Transcription factors that regulate the expression of neuronal genes remain incompletely understood. The focus of my research has been on the function a novel helix-loop-helix transcription factor, Freud-1, which is implicated in the regulation of the 5-HT1A receptor and is genetically linked to non-syndromic mental retardation. Initially, I have characterized the repressor activity of a novel long isoform of human Freud-1 and shown that it is the major isoform expressed in human cells, and that it binds and represses at a specific DNA element in the human serotonin 1A receptor promoter. I have further identified a new negative regulatory DNA element in the second intron of the dopamine-D2 receptor gene and shown that Freud-1 binds to this element *in vitro* as well as in chromatin, and mediates repression by this element. Importantly, specific depletion of Freud-1 protein levels resulted in upregulation of dopamine-D2 receptor expression. Additionally, I identified and characterized a functional polymorphism in the dopamine-D2 receptor gene that is located proximal to the repressor element, and which attenuated Freud-1 binding and activity by half. This functional dopamine-D2 receptor gene polymorphism was genetically linked to a well-studied Taq1A polymorphism of unknown function that is associated with addictive disorders. Association analysis did not reveal an association of this polymorphism in patients with major depressive disorder or schizophrenia compared to normal subjects. In summary, I have identified Freud-1 as an important transcription factor involved in regulation of dopamine-D2 receptor gene expression. Altered regulation of Freud-1 could lead to alterations in dopamine-D2 and serotonin 1A receptor expression that could be implicated in mental illness, as well as in cognitive development.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

λ	wavelength
χ^2	chi-squire
µl	microlitter
[^{11}C]	carbon-11
[^{3}H}	tritium
°C	degrees Celsius
µm	micrometer
^{125}I-MPPI	4-(2'-Methoxy-phenyl)-1-[2'-(n-2"-pyridinyl)-p-iodobenzamido]-ethyl-piperazine
3D	three dimensional
5-HT	serotonin, 5-hydroxytryptamine
5-HT1A	serotonin 1A
5-HT1B	serotonin 1B
5-HT1D	serotonin 1D
7TM	seven transmembrane
8-OH-DPAT	8-Hydroxy-2-(di-n-propylamino)tetralin
A7	human melanoma cell line
AC	adenylyl cyclase
ADHD	attention deficit hyperactivity disorder
ANKK1	kinase domain containing 1
anti-hFreud-1_L	anti-human Freud-1Long antibody
AP1	activator protein 1
ARX	Aristaless Related Homeobox
ATRX	alpha thalassemia/mental retardation syndrome X-linked
BAF250	Brg-1 associated factors
bHLH	basic helix-loop-helix
bp	base pair
Brg1	Brahma-related gene1
Brm	Brahma
C2	protein kinase C conserved region 2
Ca^{2+}	calcium ion
CaMK	Ca^{2+}/calmodulin dependent protein kinase
cAMP	3'-5'-cyclic adenosine monophosphate
CC2D1A	coiled-coil and C2 domain containing 1A
cDNA	complementary DNA
CHIP	Chromatin Immunoprecipitation
CIHR	Canadian Institute of Health Research
CMV	cytomegalovirus promoter
CNS	central nervous system
CO_2	carbon dioxide
COMT	catechol-O-methyltransferase
coREST	REST co-repressor
CPM	counts per min
CRE	cAMP-responsive element

CREB	CRE binding protein
CRM1	chromosome region maintenance 1
CtBP-1	C-terminal binding protein 1
C-terminal	carboxy-terminal
D2-DRE	D2-Dual Repressor Element
D2L	DRD2-long isoform
D2S	DRD2-short isoform
DA	dopamine
DAAO	D-amino acid oxidase
DAG	diacylglycerol
DAT	dopamine transporter
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DEAF-1	deformed epidermal autoregulatory factor 1
DM14	*Drosophila melanogaster* 14 domain
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DOPA	L-3,4-dihydroxyphenylalanine
DRD2	dopamine-D2 receptor
DRE	Dual Repressor Element
DRRF	dopamine receptor regulating factor
DSM-III-R	Diagnostic and Statistical Manual of Mental disorders, 3rd edition
DSM-IV	Diagnostic and Statistical Manual of Mental disorders, 4th edition
DTNBP1	dysbindin
DTT	dithiothreitol
E12	embryonic day 12
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EMSA	Electrophoretic Mobility Gel Shift Assay
ER	estrogen receptors
ERE	estrogen response element
ERK	extracellular signal-regulated kinase
ETS	E26 transformation-specific domain
FCS	Fetal Calf Serum
FEV	fifth ewing variant
FGF8	fibroblast growth factor 8
FRE	5'-Repressor Element
Freud-1	Five Prime Repressor Under Dual Repression Binding Protein 1
Freud-1$_L$	long isoform of Freud-1
Freud-1$_S$	short isoform of Freud-1
g	acceleration due to gravity
Gα	alpha subunit of G-protein
G$\beta\gamma$	beta gamma subunit of G-protein
G418	Geneticin disulfate

GAP	GTPase activating protein function
GAPDH	glyseraldehyde-3-phosphate dehydrogenase
GATA-3	Gata binding factor-3
Gdi1	GDP Dissociation Inhibitor 1
GDP	guanosine diphosphate
GFP	Green Fluorescent Protein
Gi	inhibitory G-protein
GPCR	G-protein coupled receptor
G-proteins	heterotrimeric guanine nucleotide binding regulatory proteins
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GRK	G-protein-coupled receptor kinases
Gro/TLE	Groucho/transducin-like Enhancer of split
Gs	stimulatory G-protein
GTP	guanosine triphosphate
h	hour
HAT	Histone Acetyl Transferase
HCl	hydrochloric acid
HDAC	Histone Deacetylase
HEK293	human embryonic kidney cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HES1	Hairy/Enhancer of Split 1
HLH	helix-loop-helix
HTR1A	5-HT1A receptor gene
IκB	inactivation of its inhibitor
IP3	inositol-1,4,5-trisphosphate
IPTG	isopropyl-β-D-thiogalactopyranoside
K	potassium
kb	kilobases
KCl	potassium chloride
kDa	kilo Daltons
KO	knockout
L	litre
LD	linkage disequilibrium
LMB	Leptomycin B
LMO4	LIM-only 4
Lmx1b	LIM homeobox transcription factor 1 β
MAZ	MYC-associated zinc finger protein
MDD	Major Depressive Disorder
MeCP2	Methyl CpG Binding Protein 2
MEKK	MAP kinase kinase
MEM	Minimum Essential Medium
mFreud-1	mouse Freud-1
$MgCl_2$	magnesium chloride
min	minute
ml	millilitre

mM	millimole
MR	Mental Retardation
MR	mineralocorticoid receptor
MRE	mineralocorticoid response element
mRNA	messenger RNA
MRT3	mental retardation, autosomal recessive 3
N	sample size (number)
Na_2HPO_4	disodium hydrogen phosphate dodecahydrate
NaCl	sodium chloride
NCBI	National Center for Biotechnology Information
NES	nuclear export sequences
NF-κB	Nuclear Factor-kappaB
NG-108	mouse/rat neuroblastoma x glioma hybrid cell line
NGF	nerve growth factor
NIH/3T3	embryonic fibroblast cells
Nkx2.2	NK2 transcription factor related, locus 2
NLS	nuclear localization signal
nM	nanomolar
NMDA	N-methyl-D-aspartate
NP-40	Nonidet P-40
NRG1	neuregulin-1
NRG1	neuregulin-1
NRSE	neuron-restrictive silencer element
NRSF	neuron-restrictive silencer factor
NSMR	non-syndromic mental retardation
N-terminal	amino-terminal
NUDR	nuclear DEAF-1-related
Nurr1	Nur-related factor 1
OD	optical density
OGS	Ontario Graduate Studentship
OHRI	Ottawa Health Research Institute
P	probability
P0	postnatal day 0
PAK3	p21-Activated Kinase 3
PBS	Phosphate Buffered Saline
pCMVβgal	plasmid containing CMV driving beta- galactosidase gene
PCR	Polymerase Chain Reaction
PDZ	PSD-95 (postsynaptic density)/Disc-large/ZO-1
PET	positron emission tomography
Pet-1	pheochromocytoma 12 ETS
Pfu	DNA polymerase derived from *Pyrococcus furiosus*
pGL3B	pGL3-Basic reporter vector
pGL3P	pGL3-Promoter reporter vector
pH	potential hydrogen
Phox2b	paired-like homeodomain protein 2b
PKA	protein kinase A

PKC	protein kinase C
PLC	phospholipase C
PMSF	phenylmethanesulphonylfluoride
PNS	peripheral nervous system
PR	progesterone receptor
PRE	progesterone response element
PRODH	proline dehydrogenase
PRSS12	Protease, Serine, 12
PSD-95	postsynaptic density
Pur-1	Purine binding-1
PVDF	polyvinylidene fluoride
QPCR	quantitative PCR
R	inactive receptor state
R*	active receptor state
RAR	retinoic acid receptor
RCMH	Research Center of Mental Health
RE-1	repressor element-1
REST	repressor element 1-silencing transcription factor
RGS	regulator of G-protein signalling
RNA pol II	RNA polymerase II
RNA	ribonucleic acid
RNase	ribonuclease
RT-PCR	reverse transcriptase-PCR
SD	standard deviation
SDS	sodium laureth sulfate
SDS-page	SDS-polyacrylamide gel electrophoresis
sec	second
SERT/5-HTT	5-HT transporter
SH2	Src Homology 2
Shh	sonic hedgehog
siRNA	small interfering RNA
SK-N-AS	human bone marrow neuroblastoma cell line
SK-N-SH	human neuroblastoma cell line
SMRT	silencing mediator for the retinoid and thyroid-hormone receptors
SN	substantia nigra
SNP	single nucleotide polymorphism
Sp1	specificity proteins 1
ssDNA	single stranded DNA
SSRI	selective serotonin reuptake inhibitor
STAT2	signal transducer and activator or transcription 2
SUMO	small ubiquitin-like modifier
SV40	simian virus 40
Taq	DNA polymerase derived from *Thermus aquaticus*
TBP	TATA-box binding protein
TBS	tris-buffered saline
TF	transcription factor

TFIIA	transcription factor IIA
TH	tyrosine hydroxylase
TM	transmembrane domain
TME	tris beta-mercaptoethanol buffer
TPH	tryptophan hydroxylase
TRE	3'-Repressor Element
t-test	student's t-test
U	units
v/v	volume/volume
VMAT2	vesicular monoamine transporter 2
VTA	ventral tegmental area
WAY-100635	N-[2-[4-(2-methoxyphenyl)piperazin-1-yl]ethyl]-N-pyridin-2-yl-cyclohexanecarboxamide
wHTH	winged helix-turn-helix
x	number of times
XLMR	X-linked Mental Retardation
Y-79	human retinoblastoma cell line
ZF87	zinc-finger protein 87-kDa

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opportunities to be a mentor for a number of summer students, Honours student and even post-doctoral fellows. Furthermore, I am thankful for his patience and guidance. These experiences are invaluable and will stay with me for the rest of my life.

THESIS FORMAT

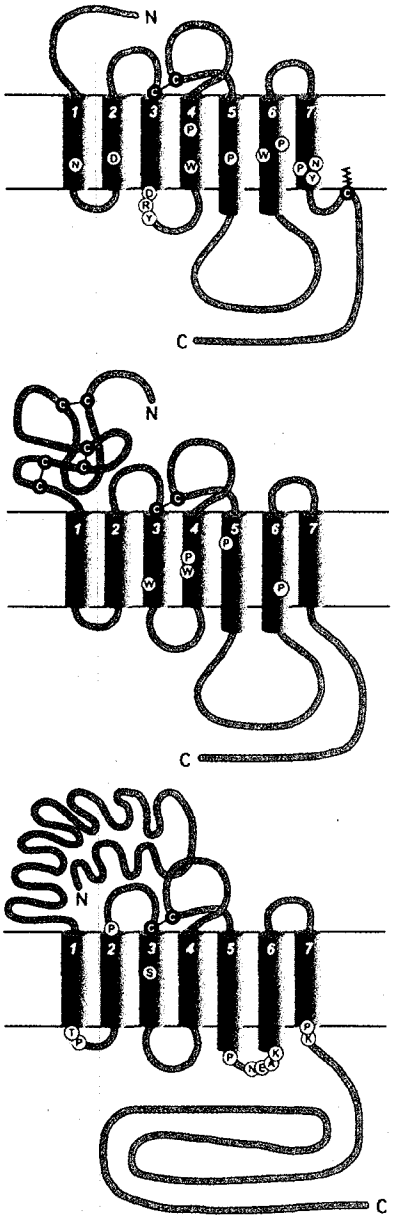
This thesis is presented in a manuscript format. Chapter I is a general introduction for my thesis project. In it, with permission from the journal, I incorporate a first author review paper entitled "The Freud-1/CC2D1A family: transcriptional regulators implicated in mental retardation" which is published in the *Journal of Neuroscience Research* (Rogaeva et al., 2007a). Chapter II is a first author paper entitled "The mental retardation gene Freud-1/CC2D1A encodes a long isoform that binds conserved DNA elements to repress gene transcription" accepted to the *European Journal of Neuroscience*. Chapter III is another first author paper entitled "Differential repression by Freud-1/CC2D1A at a polymorphic site in the Dopamine-D2 receptor gene" published in the *Journal of Biological Chemistry* (Rogaeva et al., 2007b). Chapter IV is a final first author manuscript entitled "Linkage between a novel functional *DRD2* polymorphism and the Taq1A variation: association studies in schizophrenia and depression datasets" which is so far not submitted. The final chapter V is a discussion of acquired data: its significance, study limitations and future directions in the field of mental illness.

CHAPTER I - INTRODUCTION

1.1 G-PROTEIN COUPLED RECEPTORS

G-protein coupled receptors (GPCRs) are seven transmembrane domain (TM) receptors that couple to heterotrimeric guanine nucleotide binding regulatory proteins (G-proteins) to regulate multiple physiological functions such as vision, smell, emotion and memory. Furthermore, 1-3% of the genes in the mammalian genome code for GPCRs (Gether, 2000; Palczewski et al., 2000). GPCRs are of significant interest to pharmacologists given that more than 40% of currently used drugs target these receptors (Brink et al., 2004). The first GPCR cloned was the β2-adrenergic receptor (Dixon et al., 1986). Since then, more than 1,000 different GPCRs have been identified that can be divided into three major families: Rhodopsin/β2-adrenergic receptor-like (Family A), Glucagon/VIP/Calcitonin receptor-like (Family B), and Metabotropic neurotransmitter/Calcium receptors (Family C). Biogenic amine receptors such as adrenergic, dopamine, serotonin, muscarinic, and histamine bind to the Rhodopsin/β2-adrenergic-like family A receptors (Figure I-1) (Gether, 2000). G-proteins are composed of three protein subunits: β, γ and α. The Gα can be subdivided into $G\alpha_s$ (stimulate adenylyl cyclase (AC)), $G\alpha_{i/o}$ (inhibit AC), $G\alpha_{q/11}$ (activate phospholipase C (PLC)) and $G\alpha_{12/13}$ proteins (regulate small guanosine triphosphate (GTP)-binding proteins)) (Figure I-2 and Figure I-3) (Brink et al., 2004).

GPCRs are activated by various ligands, including biogenic amines, peptides, glycoproteins, lipids, ions, nucleotides, and proteases. Ligands for receptors in Family A, are thought to bind a ligand binding pocket formed by TM domains of the receptor (Savarese and Fraser, 1992). Receptor activation causes its conformational change and



Family A. Rhodopsin/ß2 adrenergic receptor-like

Biogenic amine receptors (adrenergic, serotonin, dopamine, muscarinic, histamine)

CCK, endothelin, tachykinin, neuropeptide Y, TRH, neurotensin, bombesin, and growth hormone secretagogues receptors plus vertebrate opsins

Invertebrate opsins and bradykinin receptors

Adenosine, cannabinoid, melanocortin, and olfactory receptors.

Chemokine, fMLP, C5A, GnRH, eicosanoid, leukotriene, FSH, LH, TSH, fMLP, galanin, nucleotide, opioid, oxytocin, vasopressin, somatostatin, and protease-activated receptors plus others.

Melatonin receptors and other non-classified

Family B. Glucagon/VIP/Calcitonin receptor-like

Calcitonin, CGRP and CRF receptors

PTH and PTHrP receptors

Glucagon, glucagon-like peptide, GIP, GHRH, PACAP, VIP, and secretin receptors

Latrotoxin

Family C. Metabotropic neurotransmitter/ Calcium receptors

Metabotropic glutamate receptors

Metabotropic GABA receptors

Calcium receptors

Vomeronasal pheromone receptors

Taste receptors

Figure I-1. Three families of GPCRs.

GPCRs are sub-divided into three families. Family A has six subgroups which are characterized by highly conserved residues (*black letter in white circles*). Most of these receptors have a palmitoylated Cys at the C-terminal tail forming an additional fourth intracellular loop. Family B is characterized by a long N-terminus containing several Cys resulting in a potential disulfide bridges. However, the palmitoylation site present in

Family A is missing. The final, Family C, is characterized by a long N-terminus which is implicated in ligand binding. Other than the two Cys forming a putative disulfide bridge, Family C receptors do not have additional features except a very short and conserved third intracellular loop (Gether, 2000). (*Copyright 2000, The Endocrine Society*)

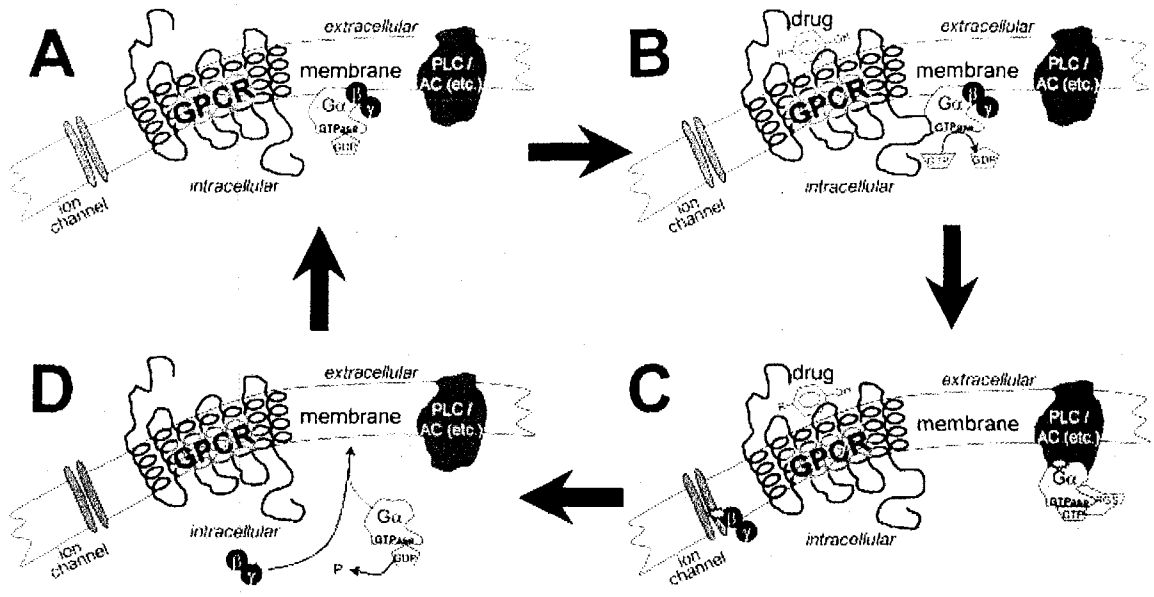


Figure I-2. Signalling of the GPCRs.

(**A**) The inactive state of the GPCRs when it is uncoupled from the G-protein. (**B**) Agonist binding changes the receptor conformation forcing receptor towards R*. The R* conformation couples to the G-protein and results in exchange of GDP for GTP on the Gα-subunit. (**C**) The Gβγ-subunit dissociates and both subunits (Gα and Gβγ) interact with downstream effectors. (**D**) Signalling is inactivated by hydrolysis of GTP to GDP using the intrinsic GTPase activity of the Gα-subunit usually catalyzed by RGS, resulting in reunion of Gα and Gβγ-subunits. The signalling is then cycled back to (A) ready for new stimuli. Abbreviations: phospholipase C (PLC); adenylyl cyclase (AC); G-protein-coupled receptor (GPCR); guanosine diphosphate (GDP); guanosine triphosphate (GTP) (Brink et al., 2004). (*Copyright 2004, Blackwell Publishing*)

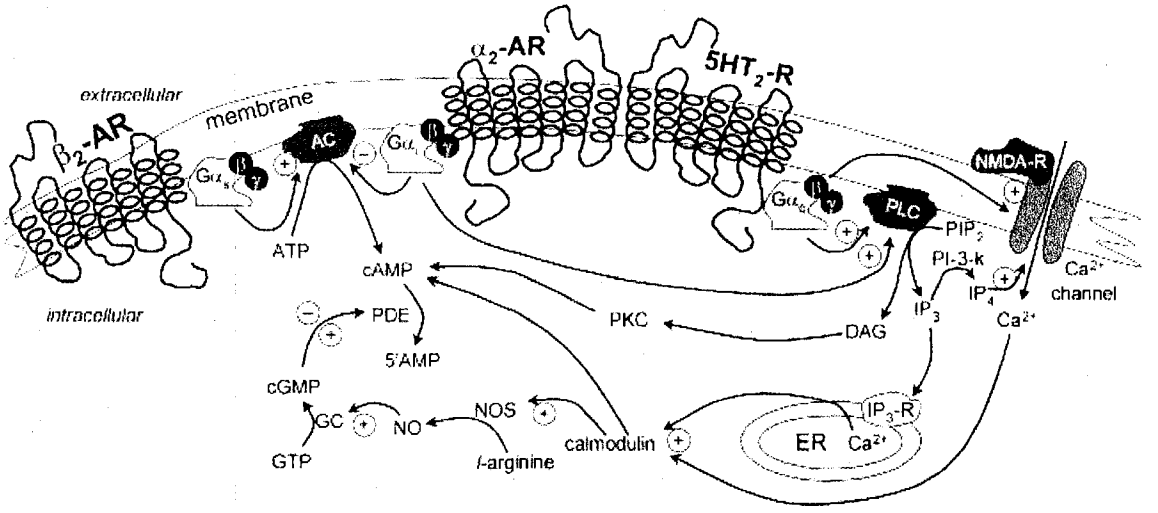


Figure I-3. A schematic representation of the GPCR signalling.

The signal transduction pathways of GPCRs (β_2-adrenergic receptor (β_2-AR); α_2-adrenergic receptor (α_2-AR); serotonin type 2 receptor ($5HT_2$-R)) and N-methyl-d-aspartate receptor (NMDA-R) and their stimulatory (+) or inhibitory effect (-) on the downstream targets: endoplasmic reticulum (ER); adenylyl cyclase (AC); phospholipase Cβ (PLC); phosphodiesterase (PDE); protein kinase C (PKC); adenosine/guanosine triphosphate (ATP/GTP); cyclic adenosine/guanosine monophosphate (cAMP/cGMP); phosphatidyl inositol biphosphate (PIP_2); inositol tri/tetra-phosphate (IP_3/IP_4); nitric oxide (NO); nitric oxide synthase (NOS) (Brink et al., 2004). (*Copyright 2004, Blackwell Publishing*)

the α-subunit of the coupled G-protein releases bound guanosine diphosphate (GDP) and binds GTP, leading to activation. The activated G-protein undergoes dissociation of the α-subunit from the βγ-dimer, both of which can then activate or inhibit downstream targets such as AC, Ca^{2+} or K^{+} channels, and phospholipases, consequently regulating second messengers such as 3'-5'-cyclic adenosine monophosphate (cAMP), inositol-1,4,5-trisphosphate (IP3), Ca^{2+} and diacylglycerol (DAG) (Figure I-3). To silence this signalling cascade, regulators of G-protein signalling (RGS) proteins with GTPase activating protein function (GAP) are recruited to catalyze intrinsic GTPase activity of α-subunit. This enhances conversion of GTP to GDP, consequently inactivating G-proteins. Receptor signalling can also be silenced in other ways, including endocytosis following phosphorylation with G-protein-coupled receptor kinases (GRK) and recruitment of β-arrestin. The receptor is then endocytosed in clathrin coated pits, which either fuse with lysosomes, consequently degrading the receptor, or are recycled back to the cell membrane after de-phosphorylation and silencing of the signalling cascade (Figure I-3) (Luttrell et al., 1999; Brink et al., 2004).

Several lines of evidence demonstrate that some receptors can continue to signal even after the G-protein is uncoupled. Studies using yeast-two hybrid, pull-down and immunoprecipitation assays examined interaction of GPCRs with signalling proteins other than G-proteins and revealed a number of interactions. As mentioned above, β-arrestins are recruited to GRK-phosphorylated receptors and allow for receptor to signal even after it has been uncoupled from the G-protein through interactions of β-arrestin and its signalling partners such as tyrosine kinase Src (Luttrell et al., 1999). Furthermore, a number of interactions with adapter protein sequences such as Src Homology 2

(SH2/SH3; (Karoor et al., 1998)), PSD-95 (postsynaptic density)/Disc-large/ZO-1 (PDZ domains; (Xu et al., 1998)) and polyproline regions (Tu et al., 1998) have been shown to link GPCRs with signalling pathways other than G-protein dependent pathways. This level of complexity of GPCR signalling means that >1,000 receptors interacting with only ~20 G-proteins can have wider combination of possible signalling paradigms (Hall et al., 1999).

Until recently the three dimensional (3D) structure of GPCRs was only hypothesized, but in the year 2000, the X-ray crystal structure of the GPCR rhodopsin was produced. This scientific breakthrough provides a model structure that is applicable to other GPCRs to examine the roles of specific amino acids in receptor signalling and ligand binding (Palczewski et al., 2000). In general, there is high amino acid conservation between GPCRs in each given subfamily. For example, there is 40-63% amino acid identity between members of the 5-HT1 subfamily (Hoyer et al., 2002). The structure and signalling properties of GPCRs is further complicated by their ability to heterodimerize and homodimerize. This property could not only produce efficient signalling of the receptor, but might also create sites for new drugs (Brink et al., 2004).

GPCRs are found in equilibrium between active (R*) or inactive (R) state, a property that has been illustrated by characterization of partial or inverse agonists. Initially, Castillo and Katz in 1957 proposed a concept that a receptor undergoes a conformational change following ligand binding (Del Castillo and Katz, 1957). Since then a number of molecules have been shown to be strong agonists (e.g. (-)-quinpirole (Newman-Tancredi et al., 1999), 5-hydroxytryptamine (5-HT; (Newman-Tancredi et al., 1992)), 8-Hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT; (Newman-Tancredi et al.,

1998a)) for serotonin 1A (5-HT1A) receptor) which bind primarily to the R* state of the receptor. In contrast, partial agonists are proposed to bind weakly to R and strongly to R* (e.g. ziprasidone for 5-HT1A receptor (Newman-Tancredi et al., 1998b)) both forcing the equilibrium towards the active state. On another hand, inverse partial agonists bind strongly to the R and weakly to R* and inverse strong agonists bind mainly to R resulting in inactivation of the receptor if it already has significant constitutive activity otherwise this interaction will be unnoticed. Finally, antagonists (e.g. N-[2-[-(2-methoxyphenyl)piperazin-1-yl]ethyl]-N-pyridin-2-yl-cyclohexanecarboxamide (WAY-100635) for 5-HT1A receptors (Newman-Tancredi et al., 1998a)) bind to both R and R* states with the same efficacy and have no effect on their own other than competition for the receptor with either agonists or inverse agonist, consequently reversing their effects (Figure I-4) (Brink et al., 2004). To further complicate this model it is suggested that more than one active state of the receptor could be present (R* and R**) depicted by selectivity of one receptor for different types of G-proteins and consequently downstream signalling cascades (Berg et al., 1998).

A number of GPCRs are not only drug targets, but are implicated in various disorders. An amino acid substitution mutation identified in 1992 in rhodopsin leads to constitutively active receptor and consequently autosomal dominant retinitis pigmentosa (Robinson et al., 1992). The disease implications of two neurotransmitter systems (dopamine and serotonin), with focus on dopamine-D2 and serotonin 1A receptors will be discussed in detail. These receptors are of interest because, in addition to their prominent roles as post-synaptic receptors to mediate neurotransmitter actions, they also

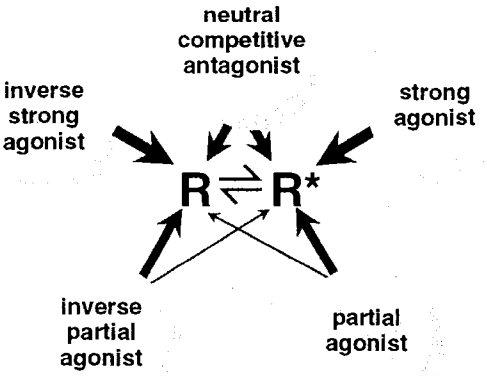


Figure I-4. A schematic representation of two-state receptor model.

An effect of drugs (strong agonists, partial agonists, neutral competitive antagonists, inverse agonists, and inverse partial agonists) on the two-state receptor model. Depicted is the constant equilibrium between inactive (R) and active (R*) receptor conformations in addition to the preference and the effects of drugs on the receptor state (Brink et al., 2004). (*Copyright 2004, Blackwell Publishing*)

function as presynaptic autoreceptors on serotonin and dopamine neurons, respectively. A number of GPCRs are located at both presynaptic and postsynaptic sites. GPCRs that are located at the presynaptic sites are sensitive to neurotransmitter released by that same neuron and are referred to as "autoreceptors" that regulate neuron firing through negative feedback mechanism (Raiteri, 2001). Thus, autoreceptors regulate the neurotransmission of the entire system, affecting multiple brain regions and physiological processes.

In summary, GPCRs, despite their diversity, retain many common properties including coupling to G-proteins, structural similarity, and similar mechanisms of desensitization; however, the specific ligands, signals and desensitization properties vary between receptor subtypes.

1.1.1 Serotonin system

The monoamine neurotransmitter serotonin (5-HT) was first discovered in serum in 1947, as a component that induced vasoconstriction (Rapport et al., 1947). It is synthesized from the essential amino acid L-tryptophan by the rate limiting enzyme tryptophan hydroxylase (TPH), which produces 5-hydroxy-tryptophan. The product is then decarboxylated by the aromatic L-amino acid decarboxylase (AADC) to yield 5-HT. The neurotransmitter is then taken up into storage vesicles, while non-sequestered 5-HT is metabolized to 5-hydroxyindoleacetic acid by monoamine oxidase (Figure I-5) (Boadle-Biber, 1993). 5-HT remains in storage vesicles until an action potential induces its release into the synaptic cleft where it activates post-synaptic, as well as pre-synaptic serotonin receptors by feed back mechanisms. The residual neurotransmitter is rapidly taken up by 5-HT transporter (SERT/5-HTT) to be recycled or packaged back into new

vesicles (Figure I-6) (Sibille and Lewis, 2006). The vesicular monoamine transporter 2 (VMAT2) is an essential protein for this function (Weihe and Eiden, 2000).

Serotonin receptors are found in central and peripheral nervous systems (CNS and PNS, respectively). Furthermore, a number of receptors are also located in non-neuronal tissues such as gut, cardiovascular system and blood (Hoyer et al., 2002). Serotonergic neurons are primarily found in the raphe nuclei (B1-B9), with a large number of serotonergic cell bodies clustered together in the midbrain-hindbrain raphe nuclei. These ~20,000 neurons (in rat) project to almost all the brain regions: amygdala, hippocampus, cerebral cortex, hypothalamus, striatum and the spinal cord (Figure I-7) (Tork, 1990; Hendricks et al., 1999). The rostral raphe nuclei and their projections are implicated in regulating cerebral blood flow, sleep and mood. The caudal raphe nuclei send descending projections to the spinal cord and are involved in cardiovascular function, nociception and movement (van Doorninck et al., 1999).

The serotonin system has been implicated in a number of mental illnesses, including depression, anxiety, social phobia, schizophrenia, obsessive compulsive disorder and panic disorder. This implication is based in part on therapeutic efficacy of drugs that selectively alter serotonin neurotransmission, such as selective serotonin reuptake inhibitor (SSRI) in clinical treatment of the above-mentioned diseases (Breier, 1995; Bouwer and Stein, 1998; Gorwood, 2004). Furthermore, migraine, hypertension, pulmonary hypertension, eating disorders, vomiting and irritable bowel syndrome are also thought to be serotonin system dependent (Hoyer et al., 2002). Serotonin was also implicated in these disorders by studies of the phenotypes of animal models lacking (Holmes et al., 2003a) or overexpressing (Kusserow et al., 2004) genes coding for

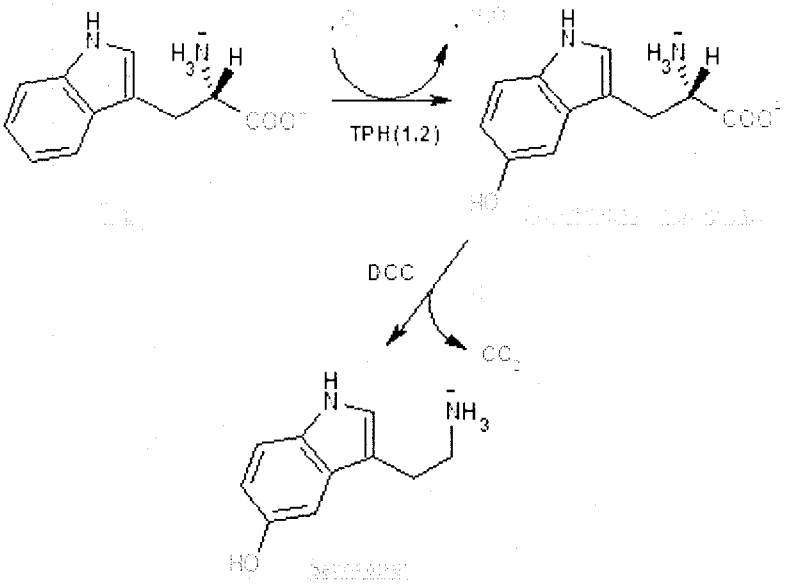


Figure I-5. Serotonin synthesis.

L-tryptophan (**Trp**) is converted to 5-hydroxy-tryptophan with tryptophan hydroxylase (**TPH**) following which the product is converted to serotonin using aromatic L-amino acid decarboxylase (**DDC**). (*No copyright required adopted from Wikipedia - http://en.wikipedia.org/wiki/Main_Page*)

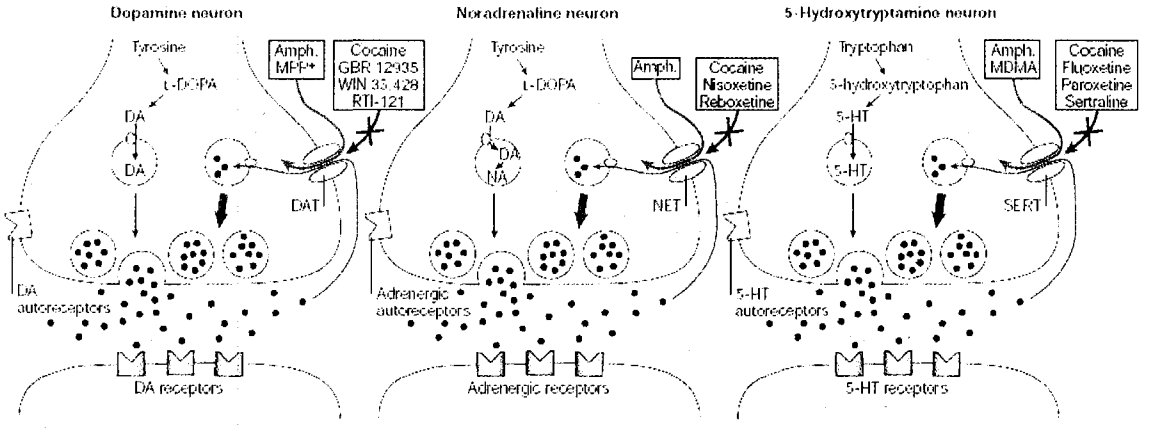


Figure I-6. A diagram of dopamine, noradrenaline and serotonin synaptic terminals. Presynaptic localization of transporters is demonstrated where they are involved in terminating neurotransmission and the neurotransmitter storage. A number of pharmacological agents which alter transporter activity are shown (amphetamine (Amph.)). Abbreviations: dopamine (DA); dopamine transporter (DAT); L-3,4-dihydroxyphenylalanine (L-DOPA); 5-hydroxytryptamine (5-HT); 1-methyl-4-phenylpyridinium (MPP^+); (+)-3,4-methylenedioxymethamphetamine (MDMA); noradrenaline (NA); noradrenaline transporter (NET); 5-HT transporter (SERT) (Torres et al., 2003). (*Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience (Torres et al., 2003), copyright (2003)*)

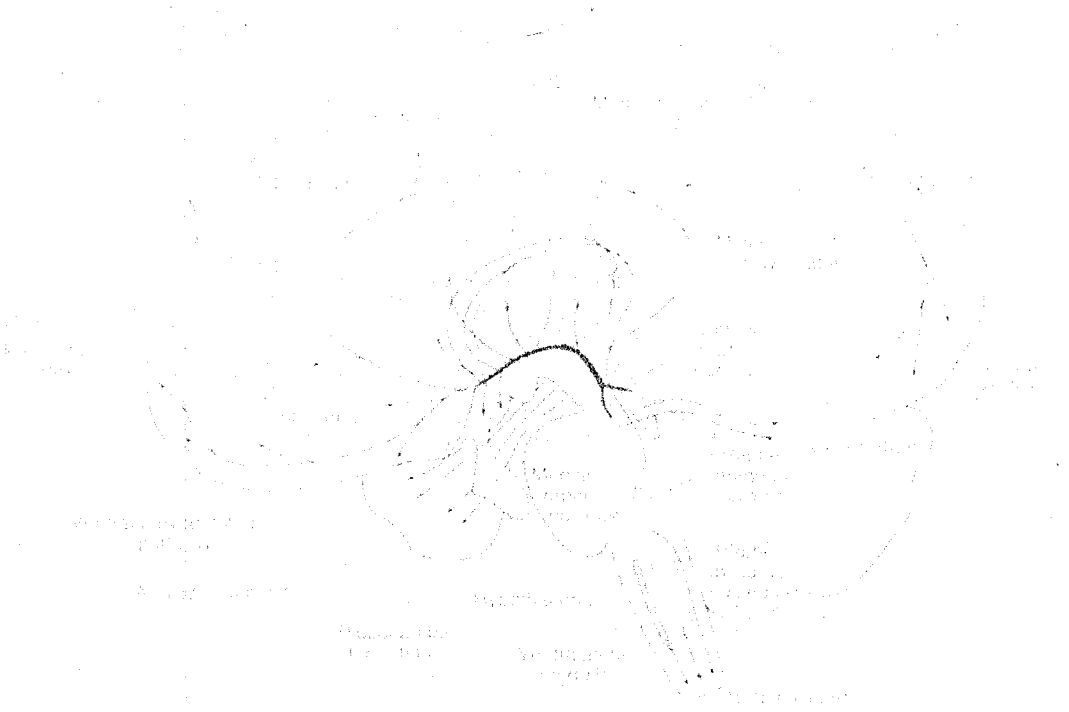


Figure I-7. Serotonergic projections in the brain.

The schematic representation of the serotonergic projections in the human brain. The ascending projections originate in the dorsal and median raphe nuclei and innervate the diencephalon in the median forebrain bundle. These projections enter the limbic system, hypothalamus, striatum and cerebral cortex. The descending projections originate in the raphe magnus, obscurus nuclei and ventrolateral medulla and project primarily to the spinal cord (Tork, 1990). (*Copyright 1990, granted by Blackwell publishing*)

proteins involved in the serotonin system. For example, KO animals for transcription factor pheochromocytoma 12 ETS (Pet-1), which is involved in the development of the serotonergic system, have an 80% deficiency in 5-HT neurons and display aggression and anxiety phenotypes, implicating the serotonin system in these behavioural changes anxiety phenotypes (Hendricks et al., 2003). Furthermore, positive association studies between polymorphisms and the genes encoding proteins of the serotonin system have given additional support for the implication of the serotonin system in a number of psychiatric illnesses (Eddahibi et al., 2003; Borroni et al., 2005; Hu et al., 2006; Park et al., 2006). More recently, positron emission tomography (PET) studies have provided a link between altered receptor expression in living human subjects and disease states such as panic disorder (Neumeister et al., 2004).

The development of the 5-HT neurons is regulated in part by a number of signalling molecules produced by the floor plate and the notochord including sonic hedgehog (Shh) and fibroblast growth factor (FGF8 and FGF4). Shh and FGF8 are involved in determining the rostral hindbrain 5-HT neurons following the induction of FGF4 (Ye et al., 1998). Furthermore, Gli2 and Gata binding factor-3 (GATA-3) and GATA-2 transcription factors are implicated in the development of the serotonergic system. Gli2 mutant animals do not form midbrain, hindbrain and spinal cord. They also demonstrate 50% reduction in 5-HT neurons, which were abnormally located to the ventral midline comparative to the bilateral clusters in wild-type animals (Matise et al., 1998; Craven et al., 2004). The absence of GATA-3 reduced the number of 5-HT positive neurons in the caudal raphe nuclei, but had no effect on the rostral raphe nuclei. These animals also demonstrated impaired locomotor activity indirectly implicating the

serotonin system in locomotion (van Doorninck et al., 1999). In addition, two homeodomain proteins, NK2 transcription factor related, locus 2 (Nkx2.2) and LIM homeobox transcription factor 1 β (Lmx1b) are also involved in the development of the serotonin system. Nkx2.2 mutant mice do not develop caudal 5-HT neurons while serotonergic neurons in the dorsal raphe nucleus are not affected by the absence of Nkx2.2 (Briscoe et al., 1999). It has been shown that paired-like homeodomain protein 2b (*Phox2b*) is repressed by Nkx2.2, consequently promoting a 5-HT cell fate instead of a motor neuron fate (Pattyn et al., 2003). *Lmx1b* regulates TH, VMAT2, and SERT expression and is essential for formation of 5-HT system in the hindbrain (Cheng et al., 2003).

There are seven serotonin receptor families which bind 5-HT with at least 14 mammalian receptor subtypes (Figure I-8). All but the 5-HT3 receptors are putative GPCRs receptors, while the 5-HT3 receptors are ligand-gated ion channels (Derkach et al., 1989). The members of the 5-HT1 receptor family (5-HT1A, 1B, 1D, 1E and 1F) are highly homologous and differ from other families by the negative coupling to the AC through Gi/o G-proteins. The 5-HT2 receptors (5-HT2A, 2B and 3C) are $Gq/_{11}$ coupled and consequently stimulate PLC, leading to a release of intracellular Ca^{2+}. Finally, 5-HT4, 6, and 7 all activate AC through the action of stimulatory G-protein (Figure I-9), while the signalling of 5-HT5 receptors remains unclear (Hoyer et al., 2002; Bonnin et al., 2006).

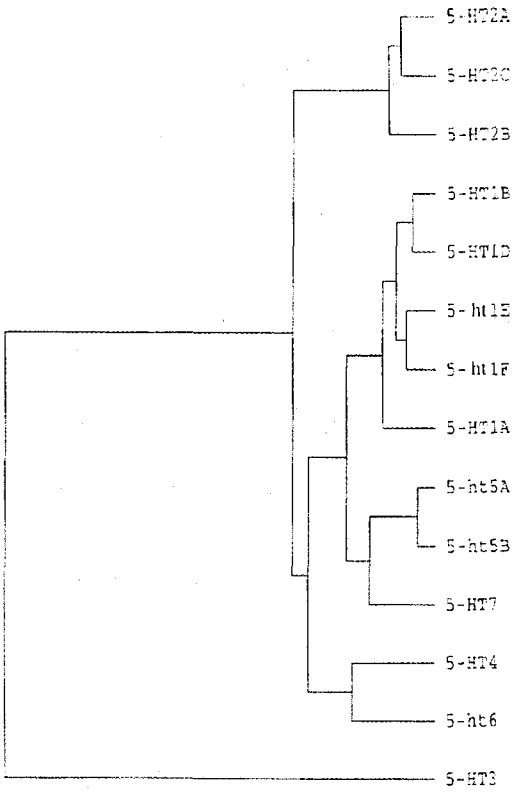


Figure I-8. Dendrogram of human serotonin receptor protein sequences.

A phylogenetic tree showing the evolution of the serotonin receptor subtypes (Barnes and Sharp, 1999). (*Reprinted from Neuropharmacology, 38, N. M. Barnes, T. Sharp, A review of central 5-HT receptors and their function, p. 1083-152, Copyright Elsevier Science Ltd., Copyright (1999), with permission from Elsevier.*)

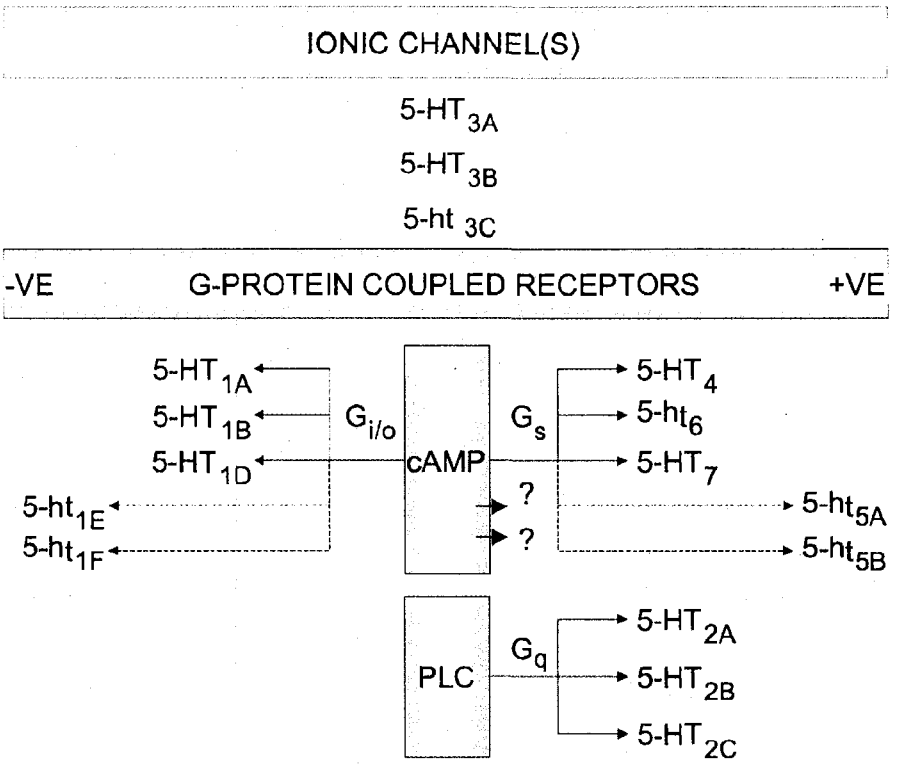


Figure I-9. Schematic representation of the serotonin receptors and their G-protein coupling.

Receptor subtypes and their coupled G-proteins. Abbreviations: cyclic adenosine monophosphate (cAMP); phospholipase C (PLC); negative (-ve); positive (+ve) (Hoyer et al., 2002). (*Reprinted from Pharmacol Biochem Behav, 71, D. Hoyer, J. P. Hannon, G. R. Martin, Molecular, pharmacological and functional diversity of 5-HT receptors, p. 533-54, Copyright Elsevier Science Inc.,, Copyright (2002), with permission from Elsevier.*)

1.1.1.1 5-HT1A receptors

One member of the 5-HT1 receptor family is 5-HT1A G-protein coupled receptor. 5-HT1A receptors signal through Gi/Go proteins to inhibit AC and calcium channels, in addition to activating potassium channels to reduce neuronal firing and neurotransmitter secretion (Albert and Lemonde, 2004; Lanfumey and Hamon, 2004; Rogaeva et al., 2007a). The human and rat 5-HT1A receptors were initially cloned in 1988 and 1990 respectively (Fargin et al., 1988; Albert et al., 1990; Fujiwara et al., 1990). The intronless gene encoding the 5-HT1A receptor is *HTR1A* (5-hydroxytryptamine (serotonin) receptor 1A) and is located at position 2q16 in *Rattus norvegicus*, 13 D1; 13 58.0 cM in *Mus musculus* and 5q12.2 in *Homo sapiens* (NCBI database and (Melmer et al., 1991)). The human *HTR1A* encodes a 421-amino acid protein. The ligand binding site of the 5-HT1A receptor was initially identified in 1981 (Pedigo et al., 1981) and since then, due in part to the anxiolytic and antidepressant properties of its ligands (Robinson et al., 1990), the receptor has become one of the best characterized serotonin receptors (Barnes and Sharp, 1999).

The neurotransmission of 5-HT1A receptor positive neurons is highly regulated by 5-HT1A receptor activity, protein expression and localization. 5-HT1A receptors are found at presynaptic sites on the cell body and dendrites of serotonin neurons (Verge et al., 1985) where they negatively regulate neuronal firing (Hjorth and Auerbach, 1994). The function of these receptors is dependent on their cell surface expression; hence receptor desensitization is an important regulatory mechanism. Prolonged activation of the receptors induces their desensitization and internalization and prevents the response to released neurotransmitter (Le Poul et al., 1995; Riad et al., 2001). Consequently, new

receptors would have to be *de novo* transcribed and translated from the *HTR1A*, transcription of which is tightly regulated (Parks and Shenk, 1996; Meijer et al., 2000; Wissink et al., 2000; Wissink et al., 2001; Czesak et al., 2006) and will be discussed in detail. Alternatively, the receptor might be resensitized by recycling back to the plasma membrane to continue signalling (Bhattacharyya et al., 2002).

1.1.1.2 5-HT1A receptor expression

Extensive work has been done to identify regions expressing 5-HT1A receptors. In initial studies, tritium ([^{3}H]) labelled 5-HT1A receptor ligands (8-OH-DPAT, WAY-100635, 5-HT) were used (Zifa et al., 1988; Laporte et al., 1994; Burnet et al., 1997). Recently PET imaging allows for visualization of receptor distribution in living humans using carbon-11 ([^{11}C]) labelled ligands (Pike et al., 1995; Cselenyi et al., 2006).

5-HT1A receptors are found on both presynaptic (soma and dendrites of raphe nuclei) and postsynaptic (forebrain regions) sites. They are highly expressed in limbic areas, such as hippocampus and lateral septum, cortical areas and raphe nuclei (Chalmers and Watson, 1991; Pompeiano et al., 1992; Palchaudhuri and Flugge, 2005). The detailed expression pattern of 5-HT1A receptors in the brain is illustrated in (Figure I-10). In addition, these receptors are also expressed in immune tissues (Mossner and Lesch, 1998) and implicated in immune and inflammatory responses (Abdouh et al., 2001). 5-HT1A receptor RNA transcripts can be detected as early as embryonic day 12 (E12) in the rat (Hillion et al., 1993), on the other hand, the immunoreactivity of 5-HT1A receptors can only be detected as early as E16 in the hippocampus (Patel and Zhou, 2005). Autoradiography using 4-(2'-Methoxy-phenyl)-1-[2'-(n-2"-pyridinyl)-p-iodobenzamido]-

ethyl-piperazine (^{125}I-MPPI; a selective 5-HT1A receptor antagonist (Kung et al., 1995)) to detect 5-HT1A receptors in mouse forebrain reveals the presence of 5-HT1A in the hippocampal neurons by E17 (Gross et al., 2002). Importantly the expression of *HTR1A* persists into adulthood (Table I-I) (Bonnin et al., 2006) where it is thought to regulate neurogenesis (Radley and Jacobs, 2002; Gordon and Hen, 2004).

1.1.1.3 5-HT1A receptors and disease implications

The signalling of 5-HT1A receptors has been implicated in establishing diverse cognitive and behavioural functions such as depression, anxiety, sleep, mood, pain, substance abuse, locomotion, sexual activity, aggression and learning (Pucadyil et al., 2005). Studies of 5-HT1A knockout (KO) animals further demonstrate the specific involvement of this receptor in cognition (Sarnyai et al., 2000) and anxiety (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998; Gross et al., 2002). Animals lacking 5-HT1A protein display an anxious phenotype, which can be rescued by induction of 5-HT1A expression in hippocampus and cortex during the critical period of P5 to P21 (Gross et al., 2002). On the other hand, transgenic mice overexpressing 5-HT1A receptors during embryonic development to P1.5 display reduced anxiety-like behaviours in adulthood (Kusserow et al., 2004). Furthermore, increased 5-HT1A receptor expression levels have been associated with elevated aggression in mice (Korte et al., 1996). Further evidence for 5-HT1A receptor involvement in anxiety comes from mouse model lacking SERT expression. The SERT KO animals also display a high anxiety phenotype (Holmes et al., 2003b) and reduced expression of 5-HT1A receptors (Li et al., 2000), and the anxiety phenotype is successfully improved by treatment with specific 5-

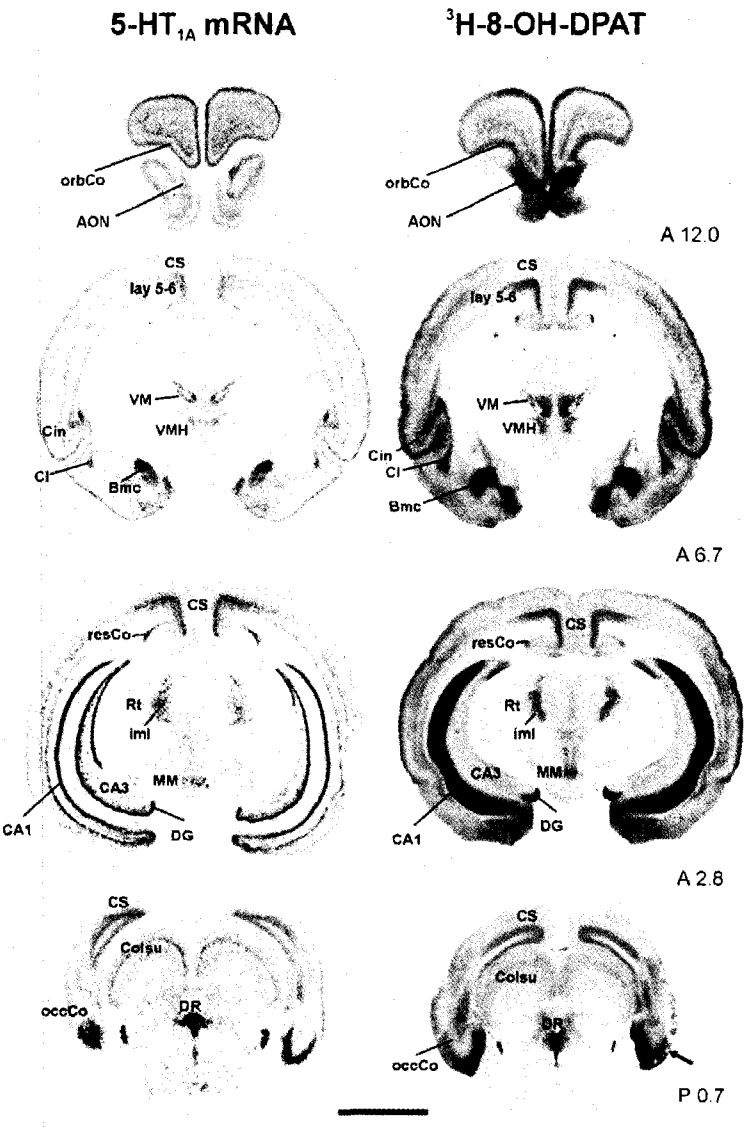


Figure I-10. The mRNA expression pattern of the serotonin 1A receptor.

In situ hybridization (left) and binding (right; 4 nM [^{3}H]8-OH DPAT) of the serotonin 1A receptor in coronal sections of the tree shrew brain. The anatomical level is depicted at the bottom right (bar=5 mm). Abbreviations: medial anterior olfactory nucleus (AON), basal magnocellular nucleus (Bmc); hippocampal regions CA1 and CA3 (CA1 and CA3), insular cortex (Cin), claustrum (Cl), colliculus superior (Colsu), striate cortex (CS), dentate gyrus (DG), dorsal raphe nucleus (DR), internal medullary lamina of the thalamus

(iml), cortical layers 5 and 6 (lay 5-6), mammillary nucleus (MM), occipital cortex (occCo), orbitofrontal cortex (orbCo), retrosplenial cortex (resCo), reticular nucleus (Rt), ventromedial thalamic nucleus (VM), ventromedial hypothalamic nucleus (VMH). (Palchaudhuri and Flugge, 2005). (*Springer and the original publisher (Cell Tissue Research, 321, 2005, pg. 162, 5-HT1A receptor expression in pyramidal neurons of cortical and limbic brain regions, M. Palchaudhuri and G. Flugge, Figure 1, copyright Springer-Verlag 2005) is given to the publication in which the material was originally published with kind permission from Springer Science and Business Media*)

Table I-I. 5-HT1A receptor expression in the mouse forebrain.

Expression of the 5-HT1A, 1B, 1D and 1F RNA in the mouse forebrain during embryonic (E14.5 and E16.5) and postnatal (P0) development (Bonnin et al., 2006). (*Reprinted from Neuroscience, 141, A. Bonnin, W. Peng, W. Hewlett, P. Levitt, Expression mapping of 5-HT1 serotonin receptor subtypes during fetal and early postnatal mouse forebrain development, p. 781-94, Copyright IBRO Published by Elsevier Ltd., Copyright (2006), with permission from Elsevier.*)

Forebrain Structure	E14.5	E16.5	P0
Telencephalon			
Amygdala (AA)	1A 1B 1D 1F	1A 1B 1D 1F	1A 1B 1D 1F
Septum (Spt)	1A	1A	1A
Olfactory tubercle (OT)		1A	1A
Striatum			
Globus pallidus (GP)	1A 1B 1D 1F	1A 1B 1D 1F	1A 1B 1D 1F
Caudate putamen (CP)	1A 1B 1D 1F	1A 1B 1D 1F	1A 1B 1D 1F
Ganglionic eminence (GE)	1F	1D	
Hippocampus (H)	1A 1F	1A 1B 1D 1F	1A 1B 1D 1F
Dentate gyrus (DG)	1A	1A 1B 1D	1A 1B 1D
Cortex			
Cortical ventricular zone (VZ/SVZ)	1F	1D 1F	
Piriform (Pir)	1A 1B 1D 1F	1A 1B 1D 1F	1A 1B 1D 1F
Layers V-VI (V-VI)			1A 1B 1D 1F
Cortical plate (CxP)	1A 1B 1D 1F	1A 1B 1D 1F	1A 1B 1D 1F
Diencephalon			
Dorsal thalamus (DTh)			
DLG	1A 1B 1D	1A 1B 1D	1A 1B 1D
VLG	1A 1D 1F	1A 1D 1F	1A 1D 1F
Paraventricular nucleus (Pvt)	1D 1F	1A 1B 1D 1F	1B 1D 1F
VL, VPm/l, Po, Vmt	1A 1B 1D 1F	1A 1B 1D 1F	1A 1B 1D 1F
Zona incerta/reticular thalamic nucleus (Zi/Rt)	1A 1D 1F	1A 1D 1F	1A 1B 1D 1F
Hypothalamus (Hy)			
Paraventricular hypothalamic nucleus (Pvh)	1A 1B 1D 1F	1A 1B 1D 1F	1B 1D
Lateral/medial preoptic area (L/MP)	1A 1B 1D 1F	1D 1F	1D
Nucleus of lateral olfactory tract (LOT)		1B	1B 1D
Medial/lateral habenula (M/LHb)	1A 1B 1D 1F	1A 1B 1D 1F	1A 1B 1D

HT1A antagonist (WAY-100635) (Holmes et al., 2003b). More recently, an *in vivo* study of patients with panic disorder using PET imaging has detected lower levels of 5-HT1A receptor binding in affected individuals compared to healthy controls (Neumeister et al., 2004). These studies emphasize the importance of proper expression of 5-HT1A receptors to establish a normal behavioural phenotype.

Some antipsychotic drugs such as clozapine, or newer antipsychotics like aripiperazole or ziprazidone, target 5-HT1A receptors in addition to dopamine, muscarinic, cholinergic and histamine receptors (Newman-Tancredi et al., 2005). It has been suggested that the partial agonist activity of clozapine on 5-HT1A receptors improves anxiety, depression, cognitive and negative symptoms of schizophrenia (Table I-II) (Miyamoto et al., 2005). In addition, 5-HT1A receptor agonists are also therapeutically successful as antidepressants (Blier and Ward, 2003).

Further evidence for a role of 5-HT1A receptors in mental illness comes from studies of single nucleotide polymorphisms (SNPs) in the *HTR1A* locus that have been associated with a number of disorders. One 5-HT1A SNP leading to an amino acid change (Arg219Leu) has been found in patients with Tourette's syndrome and is associated with impairment of receptor signalling (Lam et al., 1996; Bruss et al., 2005). In addition, an Ala50Val substitution in TM1 of 5-HT1A receptor resulted in a lack of 5-HT response (Del Tredici et al., 2004), but this polymorphism is very rare and has not been associated with disease. The SERT gene has been shown to carry a 44-bp insertion/deletion polymorphism, with the short isoform having reduced promoter activity and consequently yielding in decreased SERT expression (Collier et al., 1996). Analysis in healthy individuals of SERT transcript genotype (short vs. long), combined with PET

Table I-II. Relative receptor and neurotransmitter affinities for antipsychotics at therapeutic doses.

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Receptor	*Cloza-pine*	*Risperi-done*	*Olanza-pine*	*Quetia-pine*	*Ziprasi-done*	*Sertin-dole*	*Sulpi-ride*	*Amisul-pride*	*Zote-pine*	*Aripipra-zole*	*Halope-ridol*
D_1	+	+	+ +	−	+	+ +	−	−	+	−	+
D_2	+	+ + +	+ +	+	+ + +	+ + +	+ + + +	+ + + +	+ +	+ + + +	+ + + +
D_3	+	+ +	+	−	+ +	+ +	+ +	+ +	+ +	+ +	+ + +
D_4	+ +	−	+ +	−	+ +	+	−	−	+	+	+ + +
5-HT_{1A}	−	−	−	−	+ + +				+ +	+ +	−
5-HT_{1D}	−	+	−	−	+ + +					+	−
5-HT_{2A}	+ + +	+ + + +	+ + +	+ +	+ + + +	+ + + +	−	−	+ + +	+ + +	+
5-HT_{2C}	+ +	+ +	+ +	−	+ + + +	+ +	−	−	+ +	+	−
5-HT_6	+ +	−	+ +	−	+				+ +	+	−
5-HT_7	+ +	+ + +	−	−	+ +				+ +	+ +	−
α_1	+ + +	+ + +	+ +	+ + +	+ +	+ +	−	−	+ +	+	+ + +
α_2	+	+ +	+	−	−	+	−	−	+ +	+	−
H_1	+ + +	−	+ + +	+ +	−	+	−	−	+ +	+	−
m_1	+ + + +	−	+ + +	+ +	−	−	−	−	+	−	−
DA transporter	+ +		+ +							−	
NA transporter	+		+ +		+ +				+ +	−	
5-HT transporter					+ +					−	

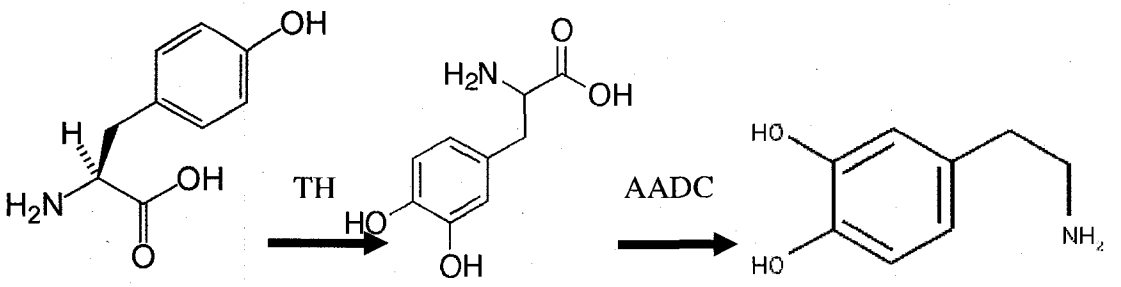
− = minimal to none; + = low; + + = moderate; + + + = high; + + + + = very high.

imaging of 5-HT1A receptor binding revealed an association between the short SERT isoform and lower 5-HT1A receptor binding in all brain regions examined (David et al., 2005). These association studies suggest links between 5-HT1A receptors and the disease states, and illustrate an important function for this receptor as a drug target and possible diagnostic parameter.

1.1.2 Dopamine system

Dopamine (DA) is a common neurotransmitter in the mammalian brain. It is synthesised from L-tyrosine by the rate limiting enzyme tyrosine hydroxylase (TH) to produce L-3,4-dihydroxyphenylalanine (DOPA), which is converted to dopamine by AADC (Figure I-11) (Vallone et al., 2000). Synthesized DA is packaged into vesicles and transported to its docking sites at the nerve terminal of the dopaminergic neurons where it is released and taken back up by dopamine transporter (DAT) to be repackaged into vesicles (Figure I-6) (Torres et al., 2003). Dopamine action is mediated by dopamine receptors, which are seven transmembrane GPCRs that are classified in two groups: D1-like and D2-like. D2-like receptors include dopamine-D2 receptor (DRD2), DRD3 and DRD4 while D1-like group includes DRD1 and DRD5. D1-like receptors couple to stimulatory G-proteins that activate AC and upregulate cAMP levels, resulting in an activation of PKA and phosphorylation of the downstream targets. The D2-like receptors inhibit AC through the actions of inhibitory G-proteins and consequently reduce cAMP production (Missale et al., 1998; Vallone et al., 2000).

Dopamine neurons project onto numerous brain regions which can be divided into four pathways: nigrostriatal, mesolimbic, mesocortical, and tuberoinfundibular. The nigrostriatal pathway regulates movement and consists of neurons originating in the substantia nigra (SN) pars compacta and projecting to the dorsal striatum. The mesolimbic pathway is implicated in mood, motivation and reward and is comprised of neurons that project from the midbrain ventral tegmental area (VTA) to the nucleus accumbens, olfactory tubercle and some limbic structures. The mesocortical pathway is implicated in learning and memory and is composed of neurons originating at VTA and



L-tyrosine **L-DOPA** **Dopamine**

Figure I-11. Dopamine synthesis.

L-tyrosine is converted to L-3,4-dihydroxyphenylalanine (L-DOPA) with tyrosine hydroxylase (TH). The DOPA is converted to Dopamine by aromatic L-amino acid decarboxylase (AADC). (*No copyright required adopted from Wikipedia http://en.wikipedia.org/wiki/Main_Page*)

innervating frontal cortex. Finally, the tuberoinfundibular pathway projects from periventricular and arcuate nuclei to median eminence of the hypothalamus, where neurosecretion of dopamine is involved in inhibiting prolactin secretion (Saucedo-Cardenas et al., 1998; Vallone et al., 2000). Therefore, the dopaminergic system is involved in the control of locomotion, cognition, reinforcement pathways, and endocrine regulation. Dopamine is a monoamine that also modulates cardiovascular function, catecholamine release and hormone secretion (Missale et al., 1998).

Alterations in neurotransmission in the dopamine system are implicated in diverse disorders including Parkinson's disease, attention deficit hyperactivity disorder (ADHD), drug abuse, depression, and bipolar affective disorder (Vallone et al., 2000; Nestler and Carlezon, 2006). A number of animal models link the dopaminergic system to these disease states. The Parkinson-like movement phenotype of knockout mice lacking DRD2 implicates these receptors in Parkinson's disease (Baik et al., 1995; Calabresi et al., 1997). Furthermore, the analysis of polymorphisms in the dopamine receptor genes and their association with disease states (e.g. schizophrenia, ADHD, mood disorders, drug abuse) provides insight into these disorders and an improved understanding of this neurotransmitter system (Swanson et al., 1998; Li et al., 1999; Grevle et al., 2000; Oliveri et al., 2000; Wong et al., 2000; Golimbet et al., 2003; Berggren et al., 2006). Other genes expressed in the dopaminergic neurons, such as TH and DAT provide a link between psychiatric disorders and the dopaminergic system (Swanson et al., 1998; Kurumaji et al., 2001). In addition, a number of drugs used clinically to improve the disease symptoms target the dopaminergic system. Some of these drugs (e.g. clozapine, cocaine) alter the expression of genes of the dopaminergic system thus providing us with a better

understanding of the proteins involved and potential mechanism for drug action (Fang and Ronnekleiv, 1999; Tejedor-Real et al., 2003).

Shh and FGF8 regulate development of dopamine neurons (Ye et al., 1998). Mature dopamine neurons express markers such as TH, Nur-related factor 1 (Nurr1), Engrailed 1/2, ptx3 and neurogenin 1 and 2 (Andersson et al., 2007). The orphan nuclear receptor, Nurr1, is required for TH induction and is implicated in the formation of the midbrain dopaminergic neurons. The Nurr1 KO animals show reduced dopaminergic neuron number and abnormal flexion-extension movements of limbs (Zetterstrom et al., 1997). In the absence of Nurr1, dopaminergic precursors expressing Ptx3 do not develop or survive, implicating Nurr1 function in the development of ventral mesencephalic dopaminergic neurons (Saucedo-Cardenas et al., 1998). Ptx3 is a homeodomain protein with a limited expression to mesencephalic dopaminergic neurons. Its expression is initiated at E11.5, which corresponds to the first appearance of the TH positive cells with the proposed function as a crucial regulator of the dopaminergic phenotype (Smidt et al., 1997). Lmx1b, which is also implicated in 5-HT neuron development, induces expression of Ptx3 in TH-positive neurons, which fail to develop in the Lmx1b KO mice (Smidt et al., 2000; Cheng et al., 2003). Expression of Ptx3 and Lmx1b marks dopamine neurons of the VTA and SN (Asbreuk et al., 2002). Finally, the Gli2 is a transcription factor, mutation of which results in a 90% decrease in the DA neurons, implicating it in dopaminergic neuronal fate (Matise et al., 1998). Although the regulation of dopamine neuron differentiation and TH expression is becoming clearer, the specific regulation of genes in the dopamine system, such as the dopamine-D2 receptor, remains relatively unexplored.

1.1.2.1 DRD2

The *DRD2* was first cloned in 1988 (Bunzow et al., 1988) and is located on chromosome 9 A5.3|9 28.0 cM in *Mus musculus*, 8q24 in *Rattus norvegicus*, and 11q23 in *Homo sapiens* (NCBI database). In *Homo sapiens* the *DRD2* gene spans over 65-kb. The gene consists of eight exons: the first exon is non-coding and is followed by a large intron (Eubanks et al., 1992) (NCBI database). DRD2 has two isoforms: DRD2-long (D2L) and DRD2-short (D2S) and it was the D2S isoforms that was cloned initially (Bunzow et al., 1988), with D2L identified shortly after (Giros et al., 1989). D2S lacks 29 amino acids at the third intracellular loop as a result of alternate splicing of exon 6 (Gandelman et al., 1991). Importantly, the third intracellular loop of the DRD2 is involved in receptor-G-protein coupling (Malek et al., 1993) and specificity (Senogles et al., 2004). The two DRD2 isoforms have been shown to couple to different second messenger systems. The D2L isoform preferentially interacts with $G\alpha_{i3}$ while D2S prefers binding to $G\alpha_{i2}$ to affect the same downstream targets (Senogles, 1994). One study has shown that the splicing of the 6^{th} intron and the ratio of the two isoform can be altered by treatment with testosterone and progesterone (Kukstas et al., 1991). Further analysis of the distribution of these isoforms in the neurons has identified the localization of D2S receptors to the presynaptic sites where they function as autoreceptors and D2L to the postsynaptic sites (Usiello et al., 2000). Dopamine-D2 receptors inhibit Ca^{2+} influx (Banihashemi and Albert, 2002), resulting in negative regulation of TH by decreasing its phosphorylation by Ca^{2+}/calmodulin dependent protein kinase (CaMK). Consequently, the DRD2 activation decreases production of DA (Sumi et al., 1991). Isoform specific KO studies revealed the involvement of D2S receptor in this negative regulation of DA

synthesis, but not the D2L receptor, supporting its presynaptic distribution (Lindgren et al., 2003).

1.1.2.2 DRD2 expression

Dopamine-D2 receptors are expressed in multiple brain regions. The analyses of the mRNA and protein expression were done using DRD2 specific probes and [^{125}I]iodosulpride binding, respectively. The DRD2 are localized to cortex, basal ganglia, septum, amygdala hypothalamus, mid/hindbrain, and retina (Bouthenet et al., 1987; Weiner et al., 1991; Levey et al., 1993; Jackson and Westlind-Danielsson, 1994).

Specifically, in the cerebral cortex, dopamine-D2 receptors and mRNA are found in all the regions analyzed but the pyriform, entorhinal, subiculum, and retrosplenial cortex (Weiner et al., 1991; Levey et al., 1993). In the basal ganglia, DRD2 is not found in the entopeduncular and subthalamic nuclei but is strongly expressed in the nucleus accumbens, caudate-putamen, and olfactory tubercle (Weiner et al., 1991). The DRD2 is widely expressed in the septum, but is not detected in some regions of amygdala (anterior, basolateral and medial nuclei), while it is highly expressed in central nucleus. The expression in hypothalamus is structure dependent, with high expression in mammillary nuclei and no expression in arcuate, periventricular, paraventricular and suprachiasmatic hypothalamus (Bouthenet et al., 1987; Weiner et al., 1991). The mid and hindbrain is enriched in DRD2 RNA, in particular the SN pars compacta, VTA, dorsal tegmental nucleus and medial parabrachial (Weiner et al., 1991). In the hippocampus the receptors are found in the lacunosum moleculare layer but not in alveus, fimbria and dentate gyrus (Bouthenet et al., 1987). Finally, the DRD2 are also located in the

cerebellum and inner and outer nuclear layer in retina (Table I-III) (Bouthenet et al., 1987; Weiner et al., 1991; Levey et al., 1993; Jackson and Westlind-Danielsson, 1994).

1.1.2.3 DRD2 and disease implications

A great deal of attention has been given to the contribution of dopamine-D2 receptors to different disorders such as Parkinson's disease, drug abuse and mood disorders. Analysis of animal models lacking or overexpressing DRD2 has provided a valuable tool to examine their contribution to the disease state. The DRD2 KO mice demonstrate reduction in spontaneous movement, abnormal gate and posture similar to the symptoms observed in Parkinson's disease (Baik et al., 1995). Examination of the synaptic plasticity in striatal neurons of these mice identified abnormalities, further supporting the DRD2 involvement in the Parkinsonian symptoms (Calabresi et al., 1997). The overexpression of the *DRD2* in the striatum of mice results in reduced working memory (Kellendonk et al., 2006). Importantly, animals with mutations in the retinoic acid receptor display reduced DRD2 levels leading to altered locomotor activity further implicating DRD2 and its regulation by the retinoic acid in Parkinson's disease (Krezel et al., 1998). Mice lacking *DRD2* expression do not demonstrate ethanol reward, implicating DRD2 in addiction (Cunningham et al., 2000). The dopamine-D2 receptors are also implicated in aggression through the observation that mice lacking D2L receptor expression demonstrate reduced aggression (Vukhac et al., 2001). Finally, DRD2 KO mice also develop pituitary tumors, implicating these receptors in antiproliferative function (Saiardi et al., 1997).

Brain region	DRD2 mRNA	DRD2 binding
Cortex		
Anteromedial prefrontal	++	++
Suprarhinal prefrontal	++	++
Piriform	-	-
Entorhinal	++	++
Dorsal endopiriform nucleus	+	+
Basal ganglia		
Caudate-putamen	+++	+++
Nucleus accumbens	+++	+++
Olfactory tubercle	+++	+++
Islands of Calleja	-	+++
Globus pallidus	sc	+
Entopeduncular nucleus	+	-
Subthalamic nucleus	-	-
Septum		
Lateral	+	++
Medial	+	++
Nuclear diagonal band	+	++
Amygdala		
Basolateral	-	-
Central	++	++
Medial	-	-
Intercalated	-	+
Hypothalamus		
Dorsal	++	+
Posterior	++	+
Ventromedial	++	+
Lateral	++	+
Zona incerta	++	+
Arcuate	-	-
Periventricular	-	-
Paraventricular	+	-
Suprachiasmatic	-	-
Lateral mammillary	++	++
Medial mammillary	++	++
Mid- and hindbrain		
Lateral habenula	++	+
SN, pars compacta	+++	+++
SN, pars reticulata	sc	+
Ventral tegmental area	+++	+++
Retrorubral field	+++	+++
Cranial nerves 3 and 6	++	++
Dorsal raphe	++	+
Locus coeruleus	±	++
Central gray	++	++
Inferior colliculus	++	+
Superior colliculus	+	+
Pontine reticular	++	+
Dorsal tegmental nucleus	+++	+++
Medial parabrachial	++	+++
Retina		
Inner nuclear layer	+	+
Outer nuclear layer	-	++

Table I-III. Binding and mRNA distribution of dopamine-D2 receptors.

Dopamine-D2 receptor mRNA in the rat brain. Symbols: uncertainty (±); low to high relative levels (+ to + + +); scattered cells (sc); substantia nigra (SN) (Adapted and modified from (Weiner et al., 1991)).

Different studies have analyzed the association of various polymorphisms found in the *DRD2* gene and non-coding region proximal to it with a variety of disorders. Despite occasionally inconsistent results of such association studies, there is strong support for the involvement of the *DRD2* in the psychiatric disorders (Grevle et al., 2000; Tan et al., 2003). One polymorphism (Taq1A; rs1800497) located 3' to the *DRD2* is associated with decreased *DRD2* gene expression as well as schizophrenia (Golimbet et al., 2003), bipolar affective disorder (Li et al., 1999), alcoholism (Berggren et al., 2006) and Parkinson's disease (Grevle et al., 2000; Oliveri et al., 2000). A different polymorphism (-141C Ins/Del) located at the promoter region affects *DRD2* transcription and has also been associated with schizophrenia (Arinami et al., 1997), alcoholism (Ishiguro et al., 1998) in some studies, while in others no association was detected (Stober et al., 1998), revealing the complexity behind interpretation of the association studies.

Another important piece of evidence for the involvement of the *DRD2* in psychiatric disorders is the observation that most antipsychotic drugs target dopamine-D2 receptors (Table I-II) (Miyamoto et al., 2005). DRD2 are antagonized by most antipsychotic drugs and the antipsychotic efficacy is also correlated with the ability of the drug to block dopamine-D2 receptors (Creese et al., 1976; Takeuchi et al., 2002). In addition, DRD2 levels are upregulated in schizophrenics (Seeman, 1992) and the receptors are found in the high affinity state (Seeman et al., 2005).

1.2 TRANSCRIPTION

In the cell nucleus, the genomic DNA is packaged into chromatin by histones. The primary level of chromatin structure is 146-bp nucleosome of DNA that is wrapped around core histones (H1A, H2B, H3 and H4) making 1.75 turns, which can be extended to 165-bp by the presence of linker histone (H1) allowing the DNA to make two turns around histones. The secondary chromatin structure is represented by the interaction between nucleosomes making the DNA more compact (Figure I-12). The chromatin can be either tightly (heterochromatin) or lightly packaged (euchromatin). The heterochromatin generally contains transcriptionaly inactive genes, unlike the euchromatin (Wegel and Shaw, 2005). Modification of histones such as hypoacetylation, di- or tri-methylation of lysine 9 at histone H3 and cytosine methylation at CpG islands results in gene silencing. In contrast, the histone hyperacetylation and di-methylation of lysine 4 at histone H3 is associated with euchromatin (Gilbert and Sharp, 1999; Greenway et al., 2007). Methyl CpG Binding Protein 2 (MeCP2) is involved in methylating DNA at CpG islands and its DNA interaction is negatively regulated by Ca^{2+}-dependent phosphorylation (Chen et al., 2003). Histone Deacetylase (HDAC) and Histone Acetyl Transferase (HAT) perform opposite functions for gene transcription, the first one deacetylating and the second one acetylating histones. Deacetylation of lysine residues results in an increased positive charge that encourages stronger histone/DNA interaction (Ogbourne and Antalis, 1998). Therefore, both HDACs and HATs affect histone-histone and histone/DNA interactions and are recruited by DNA-binding factors (Bertos et al., 2001).

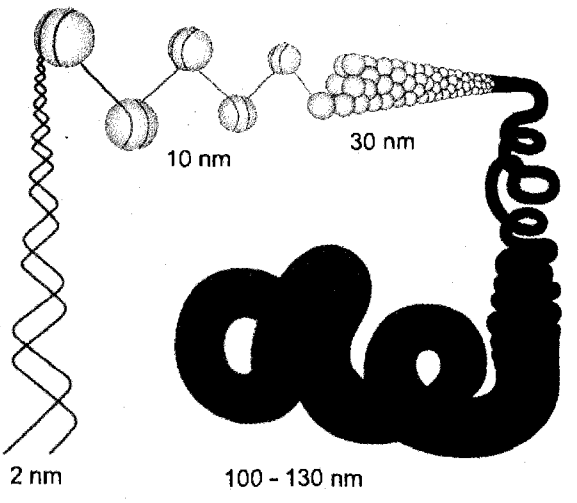


Figure I-12. Schematic representation of chromatin condensation.

The DNA helix wraps around the nucleosomes then condenses to a helical structure (Wegel and Shaw, 2005). (*Springer and the Chromosoma, 114, 2005, p. 331-337, Gene activation and deactivation related changes in the three-dimensional structure of chromatin, E. Wegel, P. Shaw, Figure 1, copyright Springer 2005 with kind permission from Springer Science and Business Media.*)

Another protein complex involved in establishing chromatin structure is the ATPase-dependent SWI/SNF chromatin remodelling complex consisting of proteins with ATPase activity such as Brahma-related gene1 (Brg1) or Brahma (Brm), acting together with Brg-1 associated factors (BAFs: 250, 155, 170, 60, 57, 53, 47) where BAF155, BAF170 and BAF47 are indispensable (Chi, 2004). There are various complexes that have been identified containing different combination of subunits (Martens and Winston, 2003). SWI/SNF has also been shown to interact with the above mentioned HDAC, HAT, methyltransferases and MeCP2 proteins (de la Serna et al., 2005; Harikrishnan et al., 2005). In addition, the SWI/SNF complex facilitates the binding of the TATA-box binding protein (TBP) to the transcription initiation site. This ATPase dependent chromatin remodelling abolishes nucleosome-mediated repression and is implicated in activation of the gene transcription (Imbalzano et al., 1994). On the other hand, it has been show to facilitate repression through its association with HDAC (Xue et al., 1998; Zhang et al., 2000) and repressor element 1-silencing transcription factor (REST) (Ooi et al., 2006). The BAF57 is thought to be the bridge between the transcription factor and SWI/SNF complex (Chen and Archer, 2005). The SWI/SNF complex achieves its functions through transient modification of histone/DNA interaction allowing for protein/DNA binding to occur (Nagaich et al., 2004).

The transcription cycle can be broken down into four stages: initiation, promoter clearance, elongation and termination (Goodrich and Tjian, 1994). The promoter activity can either be driven by TATA-box or in the case of TATA-less promoter it can be driven by an enhancer protein able to recruit transcription machinery. TATA sequence is usually located at ~30-bp away from the initiation of transcription (Gill, 1994; Javahery et al.,

1994). The TATA-box recruits TATA binding protein TBP, which initiates formation of transcriptional complex consisting of transcription factor IIA (TFIIA), TFIIB, TFIID, TFIIE, TFIIF, TFIIH and RNA polymerase II (Figure I-13) (Gill, 1994). In the case of a TATA-less promoter, a transcriptional initiator is thought to bind the PyPyA+1NT/APyPy consensus sequence (e.g. specificity proteins (Sp1)) (Javahery et al., 1994) and is able to interact with TFIID and consequently the transcription initiation complex (Figure I-13 and Figure I-14) (Pugh and Tjian, 1990). Interestingly, even before transcriptional initiation, a preinitiation complex with RNA polymerase II can be found on some promoters. In addition, the defining step in transcriptional activation was shown to be chromatin remodelling by SWI/SNF complex (Soutoglou and Talianidis, 2002). A number of regulatory DNA elements which bind a set of transcription factors can be found both proximal (Parks and Shenk, 1996) and distal (Ainscough et al., 2000) to the promoter as well as in the intronic regions (Haniel et al., 1995).

Regulation can take place at a number of steps during transcription. First, the initiation of transcription can either be inhibited or activated by the presence of transcription factors. Second, gene transcription can be inhibited by preventing completion of RNA modifications including polyadenylation at the 3'-UTR and methylation of guanidine capping at the 5'-end. Finally, intron splicing could be inhibited, preventing the RNA exit from the nucleus. Distal transcriptional repression can take place via three proposed mechanisms: physically blocking transcriptional elongation, preventing intron splicing and simply abrogating transcriptional machinery (Ogbourne and Antalis, 1998).

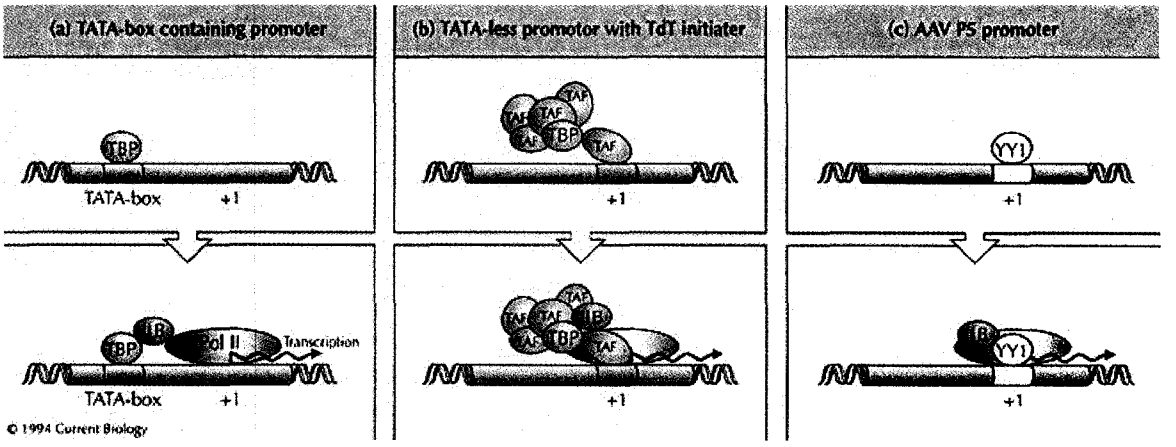


Figure I-13. Initiation of transcription at three types of promoters.

Assembly of RNA polymerase II containing transcription complexes. The transcription start site is indicated as +1 (Gill, 1994). (*This article was published in Current Biology, vol. 1, G. Gill, Transcriptional initiation. Taking the initiative, pg. 374-376, Copyright Elsevier Science Ltd. (1994).*)

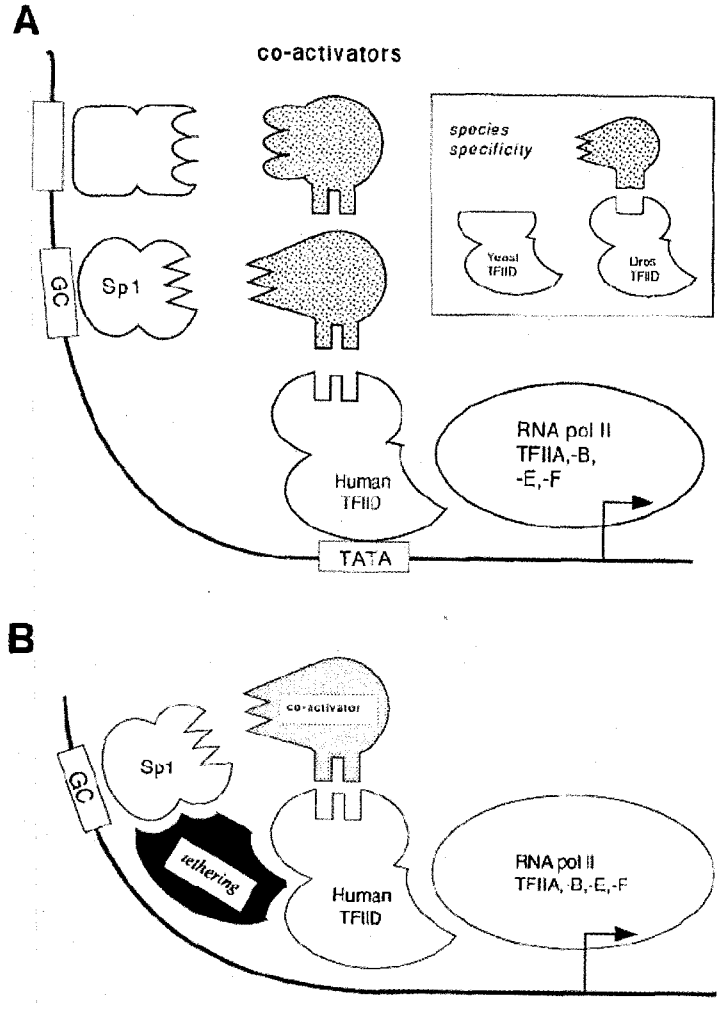


Figure I-14. Model of the transcriptional activation by Spl.

(**A**) A model proposing that specific co-activators connect different proteins into the initiation complex. (**B**) The model of Spl activation of TATA-less promoters where Spl is required to recruit the basal initiation factors (Pugh and Tjian, 1990). (*This article was published in Cell, vol. 67, B. Franklin Pugh and Robert Tjian, Mechanism of transcriptional activation by Sp1: Evidence for coactivators, pg. 1187-1197, Copyright Elsevier (1990).*)

Another way to regulate transcription is to control the cellular localization of transcription factors. Nuclear import/export is tightly regulated and a number of transcription factors are dependent on other proteins to achieve their desired localization. The nuclear pore complex on the nuclear membrane allows diffusion of proteins up to 40-kDa in size. Some proteins have a nuclear localization signal (NLS; PKKKRKV), which interacts with the pore complex resulting in energy dependent nuclear transport (Adam et al., 1990). The NLS sequences are recognized by proteins such as karyopherins, importins and transportins, which anchor proteins to the nuclear pore and aid in their translocation from the cytoplasm into the nucleus (Ossareh-Nazari et al., 1997). Furthermore, nuclear export sequences (NES; $\psi(X)_N\psi(X)_2\psi X\psi$, where ψ is any hydrophobic amino acid (L, I or V) and X is any amino acid (Craig et al., 2002)), are used to aid proteins in exiting the nucleus and thus indirectly regulating transcription by a number of nuclear export proteins. Protein localization is regulated by proteins such as chromosome region maintenance 1 (CRM1)/exportin 1. CRM1 has been shown to interact with NES of proteins thus targeting them to the nuclear pore complex and resulting in their nuclear export (Ossareh-Nazari et al., 1997). In addition, protein-protein interactions can also inhibit nuclear translocation as is the case for some nuclear receptors, which do not translocate to the nucleus until they are activated. This activation disrupts the interaction with the bound cytosolic protein (e.g. heat shock protein), which plays a role in the inhibition of this translocation (Beato, 1989).

1.2.1 Transcription factors

A great deal of research has focused on identifying and characterizing proteins and their mechanisms in transcriptional regulation. The majority of transcription factors identified can be broken down into a number of groups: basic helix-loop-helix (bHLH), zinc finger, helix-turn-helix and leucine zipper containing proteins (Tan and Richmond, 1998). Most DNA-binding proteins require co-activators or co-repressors to alter transcription. The C-terminal binding proteins (CtBP-1 and CtBP-2) are able to form homodimers or heterodimers and are co-repressors that have both unique and redundant roles (Turner and Crossley, 1998; Deltour et al., 2002; Hildebrand and Soriano, 2002). CtBP-1 is implicated in tumorigenesis and development (Shi et al., 2003). It interacts with DNA-binding transcription factors via the PxDLS amino acid sequence found at the DNA binding protein (Bertos et al., 2001) and functions in HDAC-depended and -independent fashions (Deltour et al., 2002; Shi et al., 2003). The CtBP-1 protein associates with DNA-binding proteins, histone-modifying enzymes, co-repressors and chromodomain-containing proteins (Shi et al., 2003) and its nuclear translocation is dependent on conjugation of small ubiquitin-like modifier (SUMO) at the C-terminal end (consensus: ψKXE, where ψ is a hydrophobic amino acid) (Lin et al., 2003).

A large group of transcription factors contain zinc finger protein domains involved in DNA binding. REST has nine zinc finger motifs, one of which, at the C-terminal end, is essential for the interaction with its co-repressor (coREST) (Ballas et al., 2001). REST, also known as neuron-restrictive silencer factor (NRSF), is a well studied repressor that binds to the repressor element-1 (RE-1 or neuron-restrictive silencer element (NRSE); consensus: ttCAGCACCacGGAcAGcgcC (Schoenherr et al., 1996)).

Its activity is dependent on recruitment of a variety of complexes to the promoter in order to silence neuronal (Schoenherr and Anderson, 1995) and non-neuronal gene transcription (Schoenherr et al., 1996). Among these co-repressor proteins are coREST, Sin3A and HDAC (Ballas et al., 2001). The N-terminal domain of REST associates with Sin3A and HDAC and this interaction has been shown to mediate deacetylase dependent repression of target genes (Roopra et al., 2000). In addition, it is also associated with the ATPase-dependent chromatin remodelling complex, SWI/SNF (Ooi et al., 2006). The REST/DNA interaction is sufficient to silence transcription in non-neuronal cells while in neuronal cells where REST is not expressed the coREST/MeCP2 silence transcription at the methylated DNA (Ballas et al., 2001).

Another zinc finger protein is MYC-associated zinc finger protein (MAZ; binding sequence: GGGAGGG (Bossone et al., 1992)) with the dual role of a silencer (Song et al., 2003) and an activator (Bossone et al., 1992; Kennedy and Rutter, 1992). It also functions at a number of TATA-less promoters to drive their expression (Parks and Shenk, 1996). An additional protein which binds CG-rich DNA elements is Sp1. Its common function is to enhance gene transcription (DNA recognition sequence: GGGCGG (Pugh and Tjian, 1990)), but occasionally it has been shown to repress transcription (Song et al., 2003). This protein contains three zinc finger DNA binding domains (Narayan et al., 1997) and is known to interact with DNA sequences often situated proximal to the transcription initiation start site (Parks and Shenk, 1996). Sp1 interacts with TBP-associated factors and the strength of this interaction directs the potency of the Sp1 dependent activation (Gill et al., 1994) at both TATA-less and TATA containing genes (Song et al., 2003). Other Sp1 related proteins are (Sp2, 3 and 4), where

Sp2 is the most divergent from the group. Both Sp1 and Sp3 are ubiquitously expressed in mammals (Kolell and Crawford, 2002); however, despite their similarity these proteins have sequence specificity for their DNA-targets (Koutsodontis et al., 2002). The dopamine receptor regulating factor (DRRF) is also a zinc finger transcription factor shown to interact with GC and GT-boxes thus displacing Sp1 and Sp3 transcription factors from those sites (Hwang et al., 2001). This transcription factor is expressed during development and adulthood (D'Souza et al., 2002). Zif268 is a transcription factor which interacts with GC-boxes as is the case for Sp1 and also enhances transcription (Takeuchi et al., 2002). Its expression is upregulated following DRD2 agonist treatment (haloperidol) (Nguyen et al., 1992).

GATA proteins (1-6) also belong to the zinc finger transcription factor group. They contain two zinc finger domains and form protein-protein interactions with (A/T)GATA(A/G) amino acid sequences. The KO studies revealed that all but GATA-5 are essential for embryonic development (van Doorninck et al., 1999). GATA-2 and GATA-3 are expressed in the developing brain and bind consensus sequence (A/T)GATA(A/G) (Simon, 1995). The deformed epidermal autoregulatory factor-1 (DEAF-1) protein, also known as nuclear DEAF-1-related (NUDR), is a zinc finger containing transcription factor which is not essential for its DNA binding (Michelson et al., 1999). In addition to the SAND domain, required for protein-protein interaction, the DEAF-1 protein contains NES and NLS amino acid sequences with CRM1 dependent nuclear export (Jensik et al., 2004). DEAF-1 has been shown to associate with some retinoic acid response elements (Gross and McGinnis, 1996) and the DNA binding consensus sequence for DEAF-1 is TTCGGGNNTTTCCGG (Huggenvik et al., 1998) at

which it can either negatively or positively regulate transcription (Michelson et al., 1999; Czesak et al., 2006). DEAF-1 interacts with C-terminal end of the LIM-only (LMO4) protein (Sugihara et al., 1998) and its function is implicated in development since the KO mice display defects in neural tube closure and skeletal development (Veraksa et al., 2002; Hahm et al., 2004).

Another group of transcription factors is the basic HLH proteins which are either transcriptional activators or repressors. Most bHLH proteins bind the E box (CANNTG), but there are further subdivisions into Class A activators (consensus: CAGCTG), class B activators or repressors (consensus: CACGTG) (Dang et al., 1992) and Class C repressors (consensus: CACGCG) (Garriga-Canut et al., 2001; Nakatani et al., 2004). The HLH domain of the bHLH proteins is involved in protein-protein interactions and DNA binding (Murre et al., 1989), where the basic region and the sequence proximal to it are essential for DNA sequence specificity and binding (Dang et al., 1992). Hairy/Enhancer of Split (HES1, 3, 5, 6) proteins belong to this group of transcription factors and are implicated in neurogenesis (Gratton et al., 2003; Fior and Henrique, 2005). Unlike other bHLHs, HES transcription factors bind to the DNA at the N-box (CACNAG) or the class C site (Dang et al., 1992; Hirata et al., 2001; Nakatani et al., 2004; Kageyama et al., 2007; Kita et al., 2007) and contain WRPW domain essential for recruitment of transcriptional co-repressors (e.g. Groucho/transducin-like Enhancer of split (Gro/TLE)) (Gratton et al., 2003). HES1 recruits hyperphosphorylated Gro/TLE co-repressor complex associated with chromatin remodelling to repress gene targets (Nuthall et al., 2002). HES1 and HES5 are transcriptional repressors, while HES6 exhibits positive effects on transcription through negative regulation of HES1. It forms heterodimers with

HES1 and inhibits its repressor ability. In addition, HES6 prevents interaction between HES1 and Gro/TLE co-repressor, consequently de-repressing the transcription (Gratton et al., 2003).

The Pet-1 protein, also known as fifth ewing variant (FEV) in humans, is an E26 transformation-specific (ETS) domain transcription factor which use winged helix-turn-helix (wHTH) domain to bind DNA targets consisting of a core AGGAA sequence (Donaldson et al., 1996; Peter et al., 1997; Fyodorov et al., 1998; Pfaar et al., 2002). Pet-1 positively regulates the transcription of genes of the serotonin system (Fyodorov et al., 1998; Hendricks et al., 1999). In the brain, Pet-1 is only found in serotonergic neurons, primarily in the raphe nuclei. The expression of Pet-1 precedes 5-HT by about half a day and its expression is found as early as E11 in the mouse (Pfaar et al., 2002; Hendricks et al., 2003). Its DNA recognition sequence ((G/A)(G/A)(A/C)AGGAA(G/A)T(G/A)) is found on a number of serotonergic genes (TPH, 5HTT, AADC and *HTR1A*) (Hendricks et al., 1999). Interestingly, the KO animals for *Pet-1* exhibit aggressive and anxious phenotype (Hendricks et al., 2003) similar to the *HTR1B* and *HTR1A* KO animals, respectively (Ramboz et al., 1996; Gross et al., 2002). The 5-HT system in these mice is underdeveloped with only ~20% of serotonergic neurons present (Hendricks et al., 2003).

Another very well characterized transcription factor is Nuclear Factor-kappaB (NF-κB), with its inactive form localized to the cytosol as a result of binding to the inhibitor (IκB). The inactivation of IκB by phosphorylation allows for NF-κB nuclear translocation (Figure I-15). NF-κB is made up of a combination of subunits (p52, p50,

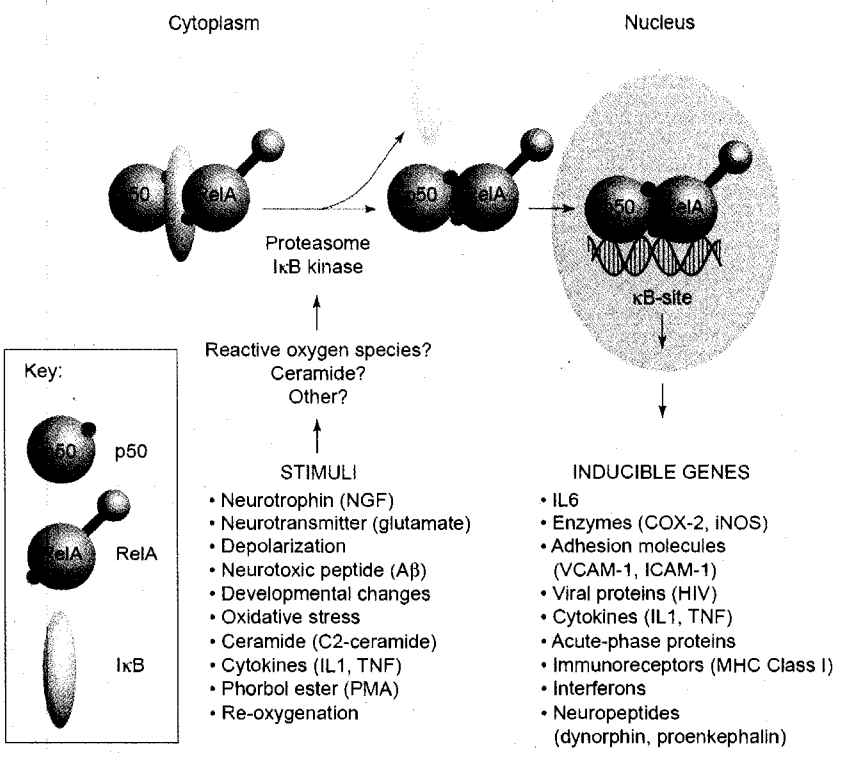


Figure I-15. Activation of Nuclear Factor-kappa B.

The Nuclear Factor-kappa B (NF-κB) is found in the cytosol as a homo- or heterodimer and is bound to the inhibitor kappa B (IκB). Stimulation leads to the phosphorylation of IκB and release of the NF-κB allowing for its nuclear translocation. In the nucleus NF-κB binds its DNA targets and activates transcription. Abbreviations: beta-amyloid (Ab); cyclooxygenase-2 (COX-2); human immunodeficiency virus (HIV); intracellular adhesion molecule-1 (ICAM-1); interleukin (IL); inducible nitric oxide synthase (iNOS); major histocompatibility complex (MHC); phorbol 12-myristate 13-acetate (PMA); tumor necrosis factor (TNF); vascular cell adhesion molecule-1 (VCAM-1) (O'Neill and Kaltschmidt, 1997). (*Reprinted from Trends Neurosci, 20, L. A. O'Neill and C. Kaltschmidt, NF-kappa B: a crucial transcription factor for glial and neuronal cell function, p. 252-8, copyright Elsevier Science Ltd., copyright 1997, with permission from Elsevier.*)

RelA (p65), c-Rel, RelB) and is found as a homo- or heterodimer. After stimuli (e.g. UV irradiation, oxidative stress or tumour necrosis factor) it translocates to the nucleus and binds at its DNA element to enhance transcription of target genes (GGGACTTTCC) (O'Neill and Kaltschmidt, 1997; Abdouh et al., 2001). Furthermore, the DNA binding ability of NF-κB is inhibited by hypophosphorylation of its p50 subunit (Kushner and Ricciardi, 1999). Importantly, NF-κB has been implicated in neuronal plasticity, neurodegeneration, neuronal development and immune responses (O'Neill and Kaltschmidt, 1997; Abdouh et al., 2001). Furthermore, genes it regulates such as *HTR1A* (Wissink et al., 2001) and *DRD2* (Bontempi et al., 2007) have also been implicated in learning and memory (Schneider et al., 1998).

A number of proteins containing the leucine zipper domain are involved in transcriptional regulation. The activator proteins (AP-1 and AP-2) are responsible for inducing gene transcription (Clark and Docherty, 1993). AP-1 is a phorbol ester-inducible protein that is composed of either jun protein homodimers or jun/fos heterodimers that bind to 12-O-tetradecanoylphorobl-13-acetate-responsive elements (TREs; TGAC/GTCA palindrome) (Sassone-Corsi et al., 1988; Masquilier and Sassone-Corsi, 1992). Another leucine zipper transcription factor is the cAMP-responsive element (CRE) binding protein (CREB) which enhances gene transcription following Ca^{2+} influx and phosphorylation at ser133 as a result of cAMP-protein kinase A (PKA) signalling (Gee et al., 2006). The phosphorylated CREB binds its DNA targets (TGACGTCA) and causes cellular effects such as neurogenesis (Zhu et al., 2004). Due to the similarity between CRE and TRE DNA elements CREB has been shown to bind TRE and compete

for the binding with AP-1, thus preventing transcriptional activation at those sites (Masquilier and Sassone-Corsi, 1992).

A number of steroid hormones are also implicated in gene transcription via their receptors. Steroid hormone receptors are divided into two groups. The first group contains the glucocorticoid, progesterone, androgen, and mineralocorticoid receptors, and the second one includes the estrogen, thyroid hormone, retinoic acid, and vitamin D3 receptors (Beato, 1989). The activation of the nuclear receptor includes both the hormone binding and consequent nuclear translocation resulting in the binding to its DNA targets as a dimer (Jensen et al., 1968). Corticosteroid hormone activates glucocorticoid receptors (GR) inducing binding to its DNA targets (glucocorticoid response element (GRE; consensus: GGTACANNNTGTTCT) or competition for the binding with other transcription factors (Beato, 1989; Ma et al., 2000). Glucocorticoid receptors exhibit both positive (Ou et al., 2001) and negative regulation of gene expression (Eberwine and Roberts, 1984; Camper et al., 1985; Zhong and Ciaranello, 1995) and associate with SWI/SNF complex (Deroo and Archer, 2001; Nagaich et al., 2004).

Estrogen is another very important hormone that activates two types of estrogen receptors (ERα and ERβ) (Mosselman et al., 1996). Upon, activation the receptors dimerize and bind to the DNA targets (estrogen response elements: EREs; classical consensus: GGTCANNNTGACC (Klein-Hitpass et al., 1986)). ERs are also known to act at non-classical EREs (Elgort et al., 1996) or directly interact with other transcription factors such as Sp1 (Krishnan et al., 1994), AP-1 (Gaub et al., 1990; Webb et al., 1995) and NF-κB proteins (Stein and Yang, 1995), all of which interact with their own DNA targets (Figure I-16). Interestingly, one target of ER is the retinoic acid receptor alpha

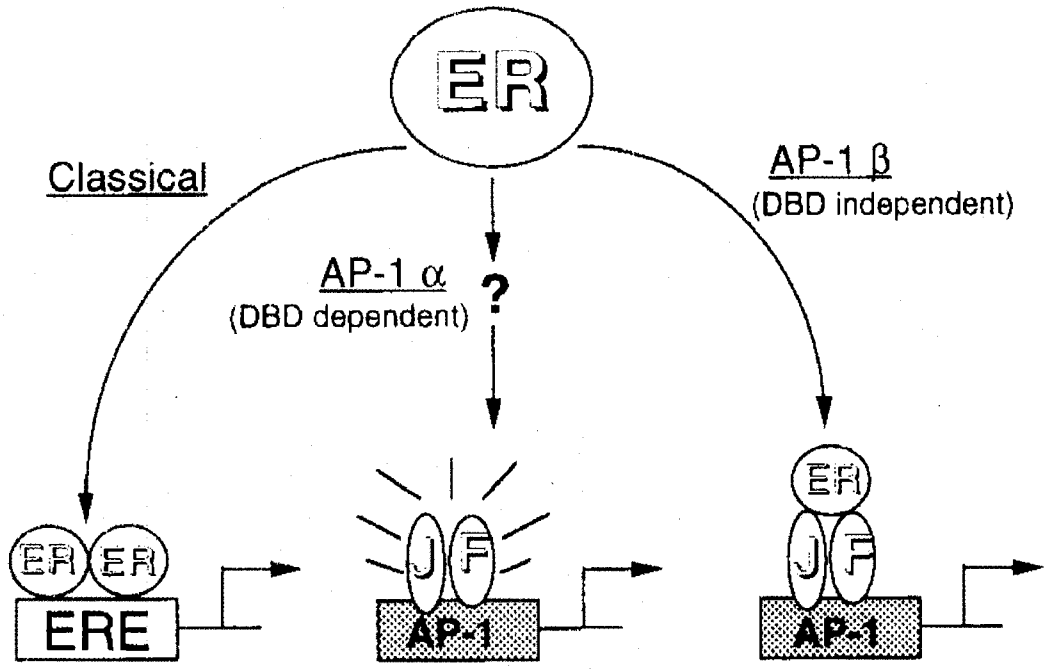


Figure I-16. Mode of action for estrogen receptor.

Estrogen receptors (ER) affect transcription at either estrogen response elements (ERE; left) or AP-1 binding sites. One mode of action at the AP-1 site involves ER interaction with an unknown target protein affecting the transcription downstream. While the other, involves ER binding to jun/fos (J/F) therefore also affecting transcription at the AP-1 site (Webb et al., 1995). (*Copyright 1995, The Endocrine Society*)

(RARα) which on its own is involved in transcriptional regulation and binds two repeats of the AGGTCA sequence with a spacer of variable length (Bahouth et al., 1998). The RARs are classified as α, β and γ and are also hormone activated nuclear receptors that exhibit both positive and negative effects on transcription. It has been shown to associate with silencing mediator for the retinoid and thyroid-hormone receptors (SMRT) co-repressor. SMRT is released in the presence of the ligand thus releasing derepression and allowing RAR to activate transcription at the DNA-targets (Chen and Evans, 1995).

The above mentioned transcription factors demonstrate overall diversity of both positive and negative regulation of a number of receptors, notably the 5-HT1A and DRD2 receptor genes. It has been proposed that this activity is in part determined by the DNA target sequence, co-factors expressed and recruitment of interacting proteins (Lefstin and Yamamoto, 1998).

1.2.2 Freud-1/CC2D1A

Characterization of transcriptional regulators of the serotonin 1A receptor gene identified the Five prime Repressor Under Dual repression binding protein-1 (Freud-1) as a negative transcriptional regulator (Ou et al., 2003). Freud-1 is also known as the coiled-coil and C2 domain containing 1A (*CC2D1A*) (Basel-Vanagaite et al., 2006). *Freud-1* is located on chromosome 19 in both *Homo sapiens* and *Pan troglodytes* and on chromosome 8 in *Mus musculus* and *Rattus norvegicus* (NCBI database).

1.2.2.1 Characterization

Characterization of the 5-HT1A promoter region detected a number of 5' regulatory sequences, one of which repressed the promoter and is called Dual Repressor Element (DRE) (Parks and Shenk, 1996; Storring et al., 1999; Ou et al., 2000). A yeast one-hybrid screen for proteins bound to the DRE identified Freud-1 protein (Ou et al., 2003). Freud-1 bound to the 5' portion of the DRE (5' Repressor Element; FRE) and repressed the promoter (Ou et al., 2003; Lemonde et al., 2004). Mammalian one-hybrid assay using Gal4-based constructs detected an intrinsic transcriptional repressor activity of Freud-1. This activity was both HDAC-dependent and -independent (Lemonde et al., 2004). These data demonstrate Freud-1 as a repressor of the *HTR1A*. In addition, a large-scale analysis of genes that activate NF-κB identified Freud-1 as an activator of NF-κB dependent transcription (Matsuda et al., 2003).

1.2.2.2 Protein Structure of Freud-1 Family

Freud-1/CC2D1A is evolutionarily conserved and similar to the related Freud-2/CC2D1B (Albert and Lemonde, 2004; Basel-Vanagaite et al., 2006). Little is known regarding the function of Freud-2; however, both Freud-1 and Freud-2 have several conserved domains: four *Drosophila melanogaster* 14 (DM14), one HLH, C2, proline-rich and coiled-coil oligomerization motifs (Burkhard et al., 2001; Rogaeva et al., 2007a). There are also a number of putative phosphorylation sites for PKA and PKC, and two CaMKII/IV sites (hFreud-1_L, Figure I-17) (Ou et al., 2003; Basel-Vanagaite et al., 2006). Initial characterization of Freud-1 detected a short isoform of mouse Freud-1 with a downstream translation initiation site (mFreud-1_S, Figure I-17) (Ou et al., 2003). The

short isoform of Freud-1 has a strong repressor activity, suggesting that the two N-terminal DM14 domains and a proline-rich domain that it lacks are not essential for Freud-1 repressor function (Rogaeva et al., 2007a). It does appear that the C2 domain is crucial for its repressor function since deleting a small 8 amino acid region abolishes its DNA-binding and repressor activity (mFreud-1_S DEL, Figure I-17) (Ou et al., 2003). The C2 domain is, therefore, essential for the repressor function of Freud-1, but additional domains such as the HLH may also play an important role in Freud-1 DNA binding and protein-protein interactions (Rogaeva et al., 2007a). A highly conserved C2 domain present in all Freud-1 orthologs suggests a Ca^{2+}-dependent regulation of Freud-1. Preliminary analysis has revealed the Ca^{2+}-mediated and ATP-dependent inhibition of Freud-1 DNA-binding and repression. This effect was reversed by calmodulin and CaMK inhibitors, indicating that Freud-1 DNA binding is CaMK-dependent (Ou et al., 2003; Rogaeva et al., 2007a).

1.2.2.3 Tissue and Subcellular Localization of Freud-1

Freud-1 RNA is expressed in low amounts in peripheral tissues compared to the CNS. The expression of Freud-1 is particularly high in cortical regions, suggesting an important function for Freud-1 in the CNS (Ou et al., 2003). *In situ* hybridization revealed high levels of Freud-1 RNA in the hippocampus and pyramidal cells of the cortex. Distribution analysis of Freud-1 protein detected Freud-1 in similar brain regions, especially where 5-HT1A receptors are abundant. Freud-1 protein and RNA are expressed in the raphe nuclei and colocalize with both serotonin and 5-HT1A receptors. This is consistent with its proposed role as a transcriptional regulator of 5-HT1A

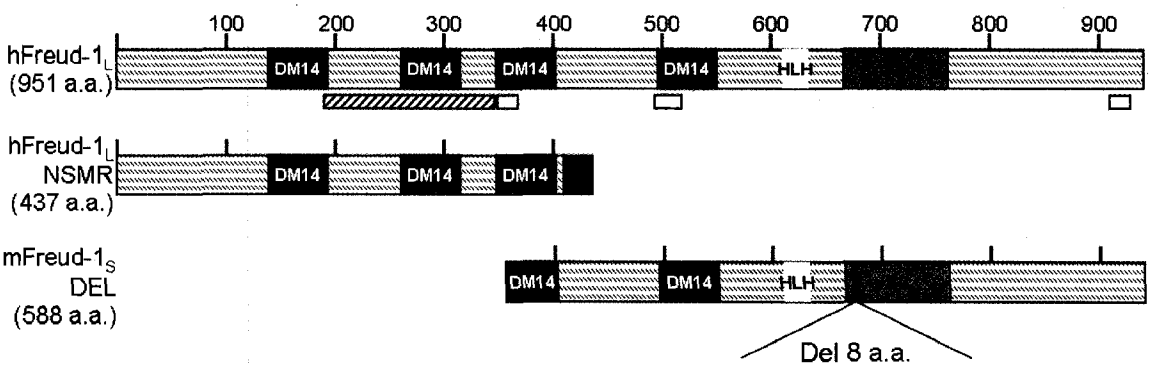


Figure I-17. Long and short isoforms of Freud-1.

"Long isoform of human Freud-1 (hFreud-1_L; NCBI accession #Q6P1N0) with four DM14 domains, one helix-loop-helix (HLH), proline-rich region (diagonal line filled box), three coiled coils motifs (white boxes) and one C2 domain is demonstrated. hFreud-1 found in the patients with NSMR is also shown (hFreud-1_L NSMR), lacking 4th DM14, HLH and C2 domains, but containing additional 30 nonsense amino acids (AACPCQQGRLCPGPAAWPGSVSGGRPALW; black box) preceding a termination codon. Lastly, the short isoform of mouse Freud-1 is demonstrated without two N-terminal DM14 domains (mFreud-1_S; NCBI accession #ABC54619). Furthermore, a location of the eight amino acid deletion which abolishes Freud-1 DNA-binding abilities is shown. Amino acid scale is depicted at the top of hFreud-1_L counted from the most upstream methionine start codon (Rogaeva et al., 2007a)." (*Copyright 2007 granted by Global Rights Department, John Wiley and Sons, Inc.*)

autoreceptor expression. Detection of Freud-1 and 5-HT1A receptor expression in the same cells indicates that Freud-1 is a regulator and not a silencer of the *HTR1A* (Ou et al., 2003). Freud-1 is also expressed in the SN and colocalizes with TH, suggesting that it may regulate transcription in dopaminergic cells (Ou et al., 2003; Rogaeva et al., 2007a).

In situ hybridization in the developing mouse brain identified Freud-1 transcript as early as E12, which persisted into adulthood (Basel-Vanagaite et al., 2006). Specifically, at E16, Freud-1 transcripts were identified in the developing cortical plate, ventricular zone progenitor cells, and in hippocampal neurons. Postnatally (P3), Freud-1 mRNA was widely expressed in hippocampal pyramidal neurons, cerebral cortex and other brain regions (Basel-Vanagaite et al., 2006). Embryonic expression of Freud-1 suggests that it may participate in development (Ou et al., 2003; Albert and Lemonde, 2004; Basel-Vanagaite et al., 2006; Rogaeva et al., 2007a).

The subcellular localization of Freud-1 implicates it in transcriptional control. Immunohistochemistry of the rat adult brain, with an anti-Freud-1 specific antibody, revealed primarily nuclear localization in dorsal raphe nucleus, hippocampus, cortex and SN. In addition, immunocytochemistry in raphe RN46A cells (derived from E13 rat raphe nucleus) and primary cultures of embryonic cortical and hippocampal cells revealed strictly nuclear distribution of Freud-1 (Ou et al., 2003). By contrast, immunocytochemistry in human U2OS osteosarcoma cells revealed primarily cytosolic distribution of Freud-1 revealing cell type dependent subcellular distribution of Freud-1 (Ou et al., 2003; Basel-Vanagaite et al., 2006). Analysis of Freud-1 protein sequence did not reveal a consensus for NLS or NES. Consequently, the shuttling mechanism of Freud-1 is yet to be determined (Rogaeva et al., 2007a).

1.2.3 *HTR1A* regulation

Transcription of *HTR1A* is regulated by a TATA-less promoter via a number of well-characterized regulatory elements and was first described in 1996 (Parks and Shenk, 1996). The promoter of 5-HT1A receptor gene is under regulation of four Sp1 sites with three proximal to the start site (-27, -77/-67) and the other two upstream of -619 at positions (-518 to -830) (Meijer et al., 2000). The activity of the *HTR1A* promoter is activated by Sp1 and MAZ at the Sp1 DNA binding sites with MAZ exhibiting a bigger activation of the promoter then the Sp1 (Parks and Shenk, 1996). The -27 Sp1 site is rich in pyrimidines (72%) and consequently could drive the transcription of the *HTR1A* by recruiting essential transcription factors for the initiation of transcription such as TAF (Gill et al., 1994; Parks and Shenk, 1996).

The promoter of *HTR1A* contains two functional NF-κB sites located at position -64 and -356 which contribute to the induction of gene transcription (Wissink et al., 2000; Wissink et al., 2001). The -64 site is highly important since its presence is correlated with strong activation of the promoter via the p65 subunit of NF-κB (Meijer et al., 2000). Interestingly, deletion of the Sp1 binding sites does not prevent Sp1/p65 activation of the promoter, indicating a regulatory role of Sp1 through the remaining second NF-κB binding site (Meijer et al., 2000). The activation of the NF-κB pathway by mitogens is linked with an increase in 5-HT1A receptor expression (Abdouh et al., 2001) and activation of 5-HT1A receptors with agonists upregulates NF-κB protein expression (Cowen et al., 1997). In addition, 5-HT1A receptor agonist treatment of lymphocytes induces nuclear translocation of NF-κB, an effect which is reversed by antagonist

treatment (Abdouh et al., 2004). These observations support an important role for this transcription factor in regulating *HTR1A* expression, at least in non-neuronal cells.

The REST and DEAF-1 proteins also exhibit regulation of *HTR1A* expression. An RE-1 element is found at position -1570 in the human gene and has been shown to repress 5-HT1A expression in a HDAC-dependent fashion. DEAF-1 regulates *HTR1A* expression via its DNA element (-1029/-998) in an orientation-independent manner (Lemonde et al., 2003; Czesak et al., 2006). PET-1 is also involved in activating the *HTR1A* promoter at its DNA element (human: -137/-127) which is interspecies conserved (Hendricks et al., 1999).

Freud-1 is a transcriptional repressor which inhibits *HTR1A* transcription. The deletion or mutation of the DRE (position: -1624 and -1598 in the human gene) results in upregulated *HTR1A* transcription. Interestingly, the mutation of the 5' portion of this element derepresses the 5-HT1A promoter in neuronal (raphe) but not in non-neuronal cells (myoblasts), suggesting that the FRE is a major regulator of *HTR1A* expression in neurons (Ou et al., 2000; Rogaeva et al., 2007a). In addition, two adjacent DREs are present in the rat and human 5-HT1A promoter, demonstrating the importance of this regulatory element in transcriptional control (Lemonde et al., 2004).

A great deal of research has focused on the negative regulation of 5-HT1A receptor expression by corticosteroid hormones. Adrenalectomy results in an increase of 5-HT1A receptor expression in the hippocampus as a result of increased transcription (Zhong and Ciaranello, 1995). Promoter analysis detected a GRE-like element (-1186 to -1145-bp) at the *HTR1A* which exhibits a negative transcriptional regulation via glucocorticoids and mineralocorticoids (Ou et al., 2001). *In vivo* evidence supports this

regulation since the hormone replacement treatment in adrenalectomized animals, where elevated levels of 5-HT1A were observed, results in reduction of 5-HT1A receptor expression following stimulation of either MR or GR (Meijer and de Kloet, 1995; Neumeister et al., 2004).

The serotonin 1A receptor gene is not only under the regulation of NF-κB, MR and GR at their own DNA-binding elements, but transcriptional control is also achieved through protein-protein interactions between these three factors. As mentioned previously, hormone receptors have been shown to directly interact with NF-κB (Stein and Yang, 1995) and transcription assays have confirmed the negative effect of MRs on the promoter of *HTR1A* at the NF-κB DNA binding sites (Wissink et al., 2000).

One well studied regulator of the *HTR1A* expression is the ER. Evidence suggests the involvement of the ER in *HTR1A* transcriptional control. A great deal of work has revealed a tissue specific regulation of *HTR1A* expression by ER where some studies have described a decrease in 5-HT1A receptor numbers and mRNA following estrogen treatment of rats (Birzniece et al., 2001) and non-human primates (Pecins-Thompson and Bethea, 1999), while others, detected an increase in 5-HT1A expression (Birzniece et al., 2001). Furthermore, combined treatments with estrogen and progesterone have shown an increase in 5-HT1A receptor expression in the hippocampus of rats (Birzniece et al., 2001) and an enhanced decrease in dorsal raphe nucleus in non-human primates (Pecins-Thompson and Bethea, 1999). Consequently, both estrogen and progesterone are involved in the regulation of *HTR1A* transcription. Given that no functional ERE and PRE have been described in the promoter of the *HTR1A*, the ER and PR may function at other binding sites such as Sp1 and NF-κB due to its above mentioned interactions with

these proteins (Krishnan et al., 1994; Parks and Shenk, 1996; Wissink et al., 2000). Estrogen has also been shown to exhibit an enhancing effect on NF-κB dependent activation of the *HTR1A* promoter (Wissink et al., 2001) and Sp1 expression is negatively regulated by estrogen (Srivastava et al., 1998). Therefore, it could have an indirect effect on the *HTR1A* expression.

Transcriptional regulation of *HTR1A* is complex and is not entirely characterized. Consequently future work is essential to understand the regulation of this disease-implicated gene.

1.2.4 *DRD2* regulation

The promoter of the human *DRD2* is incompletely characterized. The putative promoter was initially described by Arinami *et al.*, (Arinami et al., 1997). The rat *DRD2* is well studied and has a robust TATA-less, CG-rich promoter that is driven by the Sp1 transcription factor (Minowa et al., 1992, 1994; Valdenaire et al., 1994; Yajima et al., 1997; Yajima et al., 1998; Hwang et al., 2001; Dunah et al., 2002). One study has reported that rat *DRD2* transcription is driven by two promoters with two transcription start sites, one located 320-bp upstream from the 3'-end of the 1st exon and the other one an additional 70-bp upstream (Valdenaire et al., 1994).

A number of transcription factors have been shown to control the expression of *DRD2*. Initial analysis identified Sp1B (-48), Sp1A (-86) and AP-2 sites at the rat DRD2 promoter (Figure I-18). At -48, strong enhancement of the promoter was observed while at -86, the promoter was repressed as a result of the inhibited Sp1 binding to the -86 site (Minowa et al., 1992). Further analysis of the negatively regulated region (D_2NegB; -116

to -76) identified Sp3 as the factor involved in the negative regulation at the -86 site (Yajima et al., 1998). Overexpressing Sp1 specifically downregulates the DRD2 promoter activity at sequences upstream of the Sp1B site (Takeuchi et al., 2002). Another strong negative regulatory region (D_2NegA) is located between -160 and -135. This region contains a putative AP-2 binding site, but no protein-DNA interaction was detected at that site (Minowa et al., 1994).

DRD2 expression is also regulated by the retinoic acid receptor, where retinoic acid treatment upregulates DRD2 expression and promoter activity at the RARE (-68). Importantly, retinoic acid receptor KO animals demonstrate reduced levels of DRD2 mRNA in the striatum, supporting this proposed positive regulation of *DRD2* (Samad et al., 1997). In addition, RA treatment of striatal neurons resulted in upregulated DRD2 mRNA expression (Valdenaire et al., 1998). The RARE overlaps the Sp1B site, suggesting that the interaction between these proteins could contribute to the cell specific transcriptional control (Figure I-18) (Minowa et al., 1992; Samad et al., 1997). Furthermore, a GATA binding site was also identified (Samad et al., 1997) and is thought to contribute to the regulation of DRD2 tissue specific expression. The DRRF transcription factor cloned in 2001 is a negative regulator of the *DRD2* expression possibly via displacing some positive regulators from Sp1A (Hwang et al., 2001) and Sp1B DNA binding sites (Takeuchi et al., 2002).

In search for the positive regulator at the Sp1B site, MAP kinase kinase (MEKK), CaMKII and Zif268 were analyzed. MEKK uses downstream kinases as substrates some of which phosphorylate transcription factors such as c-fos and c-jun (Davis, 1995). DRD2

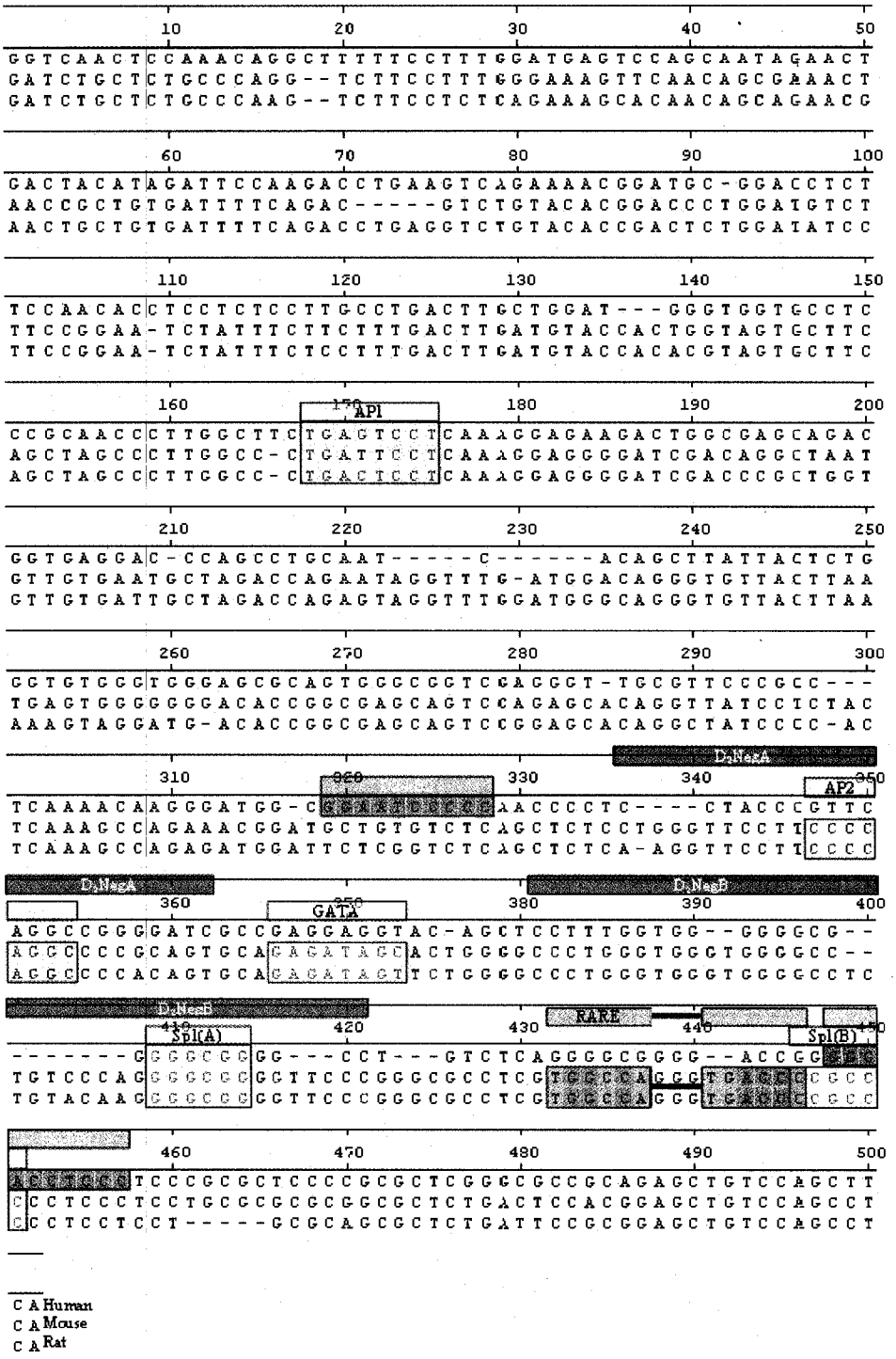


Figure I-18. Alignment of the *DRD2* promoter region in mouse, rat and human.

The identical base pairs with the human *DRD2* promoter are highlighted in yellow. Transcription factor binding sites are boxed and labeled above each site.

promoter activity was indeed upregulated following MEKK activation and the region mapped out to the Sp1B site where MEKK is proposed to affect an unknown regulator of the *DRD2* expression (Takeuchi et al., 2002). The Zif268 transcription factor is upregulated in the striatum in response to activation of the extracellular signal-regulated kinase (ERK) pathway (Sgambato et al., 1998) and was shown to induce DRD2 promoter activity at the Sp1B site. The CaMKII positively alters this activation by inducing nuclear localization of Zif268 (Takeuchi et al., 2002). ER is also able to activate *DRD2* transcription by an unknown mechanism at the same Sp1B site (Lammers et al., 1999b) which could be as a result of the activation of the ERK pathways resulting in enhanced expression of Zif268 (Sgambato et al., 1998); however, there are some disagreements in the literature where some studies described elevated DRD2 expression levels (Levesque and Di Paolo, 1991) while others state reduced DRD2 mRNA in the striatum in response to 17β-estradiol treatment (Kukstas et al., 1991; Lammers et al., 1999b). Similar effects were found with glucocorticoids where an increase in promoter activity was observed, but DRD2 mRNA levels in dorsal striatum were lower in response to corticosterone treatment (Lammers et al., 1999a).

Detailed analysis of the human DRD2 promoter (Arinami et al., 1997) revealed presence of two NF-κB sites: NF-κB1 (-407 to -398) and NF-κB2 (-513 to -504) (Bontempi et al., 2007). The activation of nerve growth factor (NGF) induces nuclear translocation of NF-κB which is essential for the observed elevation in *DRD2* expression (Fiorentini et al., 2002). Interestingly, NF-κB1 overlaps Sp1B, suggesting a potential competition for the interaction at this site between Sp1, Zif268 and DRRF transcription factors (Figure I-18).

These studies have focused on characterizing regulators of the DRD2 promoter; however, all of the attention was given to the region most proximal to the initiation of transcription, overlooking distal elements.

1.3 PSYCHIATRIC ILLNESSES

1.3.1 Mood disorders

Mood Disorders are subdivided into four groups: Depressive Disorders, Bipolar Disorders, Mood Disorder Due to a General Medical Condition and Substance-Induced Mood Disorder. Mood Disorders, also known as Affective Disorders, affect approximately 1 out 10 individuals (http://www.ontario.cmha.ca/index.asp). Major Depressive Disorder (MDD) falls in the category of Depressive Disorders together with Dysthymic Disorder and Depressive Disorder Not Otherwise Specified (American Psychiatric Association, 1994). MDD is the leading cause of disability for people between the ages of 15 and 44 (http://www.nimh.nih.gov/).

1.3.1.1 Depressive Disorders

Depression is classified by low mood, lack of motivation, anhedonia, absence of energy, and fatigue. It affects ~16% of adults in the USA (Blazer et al., 1994) and results in significant costs and family suffering (Greenberg et al., 1994). Classification and characterization of depressive disorders is complex. A great deal of research has focused on identifying the underlying mechanisms of these disorders, but a number of problems are present, preventing reproducibility of some studies. Fist, a lot of research has been done on post-mortem brain tissues with obvious limitations, one of which is protein degradation and stability. Second, a large proportion of analyzed subjects are medicated, which could skew the observed alterations. Finally, a number of different methods and ligands are utilized to examine receptor densities and proteins involved, presenting discrepancies in published work (Osterlund et al., 1999).

In a lifetime, the risk of developing MDD is 10-25% for females and 5-12% for males. MDD is characterized by depressed mood for at least two weeks or lack of interest in conjunction with a minimum of four other symptoms of depression (American Psychiatric Association, 1994). Approximately 11 out of 100,000 people die by suicide and 90% of them have diagnosable mental (primarily depressive disorder) or substance abuse disorder (http://www.nimh.nih.gov/). In addition, men are four times likely to succeed at suicide than women while women are three times more likely to report an attempt at suicide than men (http://www.cdc.gov/).

Depression is twice as likely to occur in females (8%) than males (4%), suggesting an important role for hormonal regulation or imbalance in these disease states (Osterlund et al., 1999). A number of depressive disorders such as premenstrual dysphoric disorder, postpartum depression and postmenopausal depression are responsive to hormone replacement therapy (Grigoriadis and Kennedy, 2002).

1.3.1.2 Dopamine-D2 and 5-HT1A receptors in Depression

One primary hypothesis behind depression is the "monoamine hypothesis", since a great deal of evidence implicates the DA and 5-HT systems in depression (Bunney and Davis, 1965; Ehsanullah, 1980). A high density of 5-HT1A receptors in limbic and cortical areas involved in mood regulation indicates its important role in mood regulation (Palchaudhuri and Flugge, 2005). Furthermore, expression of DRD2 in brain regions responsible for reward and motivation also implicates this neurotransmitter system in depressive disorders (Park et al., 2005).

Drugs with positive effects on depressive symptoms provide insight into the systems involved in this complex disorder. SSRIs are used to treat a number of mood disorders. Amongst them are premenstrual dysphoric disorder (Dimmock et al., 2000) and other forms of depression (Larisch et al., 1997). Dopamine agonists are also effective as treatment in some cases. Analysis of dopamine-D2 receptor number in cortical areas and striatum of patients treated with SSRIs revealed increased DRD2 levels in responders to SSRI treatment. Interestingly, the anterior cingulate gyrus is where the largest upregulation in the DRD2 was observed and this region is implicated in emotional control (Larisch et al., 1997). On the other hand, 5-HT1A receptor antagonists are also used to improve efficacy of antidepressant drugs (Artigas et al., 1994). Furthermore, antagonizing the neurokinin 1 receptor has anxiolytic and antidepressant properties in humans (Kramer et al., 1998). Interestingly, characterization of its mode of action linked it to the serotonergic system. The pharmacological data and KO studies of neurokinin 1 mice reveal downregulation of 5-HT1A autoreceptors in the dorsal raphe nucleus, resulting in increased firing of dorsal raphe neurons (Santarelli et al., 2001). The resultant antidepressant response is comparable with chronic antidepressant treatment with SSRIs where the autoreceptors desensitize disinhibiting firing of the 5-HT neurons (Albert and Lemonde, 2004).

The levels of estrogen and progesterone hormones vary during, as well as following, pregnancy and menopause and the estrogen increase during and decrease post pregnancy is associated with postpartum depression (Joffe and Cohen, 1998). Postpartum depression (Gregoire et al., 1996) and depressed mood amongst menopausal women (Zweifel and O'Brien, 1997) is successfully treated by estradiol. Late life depression is

also responsive to estradiol in combination with SSRIs (Schneider et al., 1997). Furthermore, the rat model of depression shows improvements in depressive symptoms after estradiol treatment and characterization of these animals revealed an upregulation in 5-HT2A and downregulation in 5-HT1A receptor expression, thus implicating the serotonergic system in depression (Osterlund et al., 1999). Pregnant rats also undergo depressive-like symptoms which can be eliminated by continual treatment with estradiol (Galea et al., 2001). In addition, as described previously, estrogen has been reported to exhibit both positive and negative effect on the transcription of the *HTR1A* (Birzniece et al., 2001), suggesting that the observed alterations in expression could be as a result of the changes in the transcription.

Analysis of 5-HT1A receptor expression in depressed individuals supports its involvement in the disorder. PET studies in depressed individuals identified reduced 5-HT1A receptor binding levels in midbrain raphe, limbic and neocortical areas (Drevets et al., 2000). The 5-HT1A mRNA is also reduced in dorsolateral prefrontal cortex and hippocampus of subjects with MDD (Lopez-Figueroa et al., 2004). The analysis of the dorsal raphe nucleus in MDD patients detected an increase in the 5-HT1A autoreceptor levels, consistent with reduced serotonin neurotransmission (Stockmeier et al., 1998). In addition, 5-HT1A receptors in depressed suicide subjects revealed alterations in the signalling of this receptor as the coupling to AC was reduced in the affected subjects (Hsiung et al., 2003). Importantly, 5-HT1A receptor expression post antagonist treatment revealed upregulation in receptor expression (Abbas et al., 2007). Thus, the increase in 5-HT1A receptors presynaptically leads to reduced neurotransmission which is also observed with reduced 5-HT1A receptor numbers at postsynaptic sites.

Much attention has been given to the association of SNPs with depression. Despite contradictory results, SNP association studies provide insight into potential diagnostic markers and the genetics underlying this complex and heterogeneous disorder which is moderately heritable (Levinson, 2006). One well studied polymorphism is located in the regulatory region of the *HTR1A* promoter (C(-1019)G), with the G-allele associating with MDD and reduced binding of repressor proteins (DEAF-1 and Hes5) to the promoter and subsequent upregulation of *HTR1A* expression (Lemonde et al., 2003; Parsey et al., 2006b). Furthermore, the C(-1019)G polymorphism has been shown to associate with depressive disorders in other sample groups (Huang et al., 2004). Polymorphisms in the *DRD2* also associate with depressive disorders (Li et al., 1999; Koks et al., 2006) and antidepressive effects (Suzuki et al., 2001).

Cumulatively, these observations implicate the dopamine-D2 and 5-HT1A receptors in this diverse and complex disorder.

1.3.2 Schizophrenia

Schizophrenia is a debilitating disease that affects approximately 1% of the population (Fink-Jensen, 2000) with a heritability of approximately 80% (Harrison and Owen, 2003). Family members, friends and society in general are impacted by a lifetime of disability and emotional distress caused by this disorder. Although current antipsychotic treatments have a 60% success rate, 40-60% of patients do attempt suicide and 10% complete the act, therefore more specific drugs that target key regulators of neurochemical pathways are necessary (http://www.schizophrenia.ca/). The age of onset is primarily between early teens and mid-30s. Symptoms (both positive and negative)

persist for a significant portion of one month and some symptoms can be detected throughout the following six months. The positive symptoms, such as delusions, hallucinations, disorganized speech and behaviour, represent a distortion as well as excess of normal function. On the other hand, negative symptoms such as reductions in emotional expression, clarity of thought and speech, as well as initiation of goal-directed behaviour demonstrate a lack of normal function (American Psychiatric Association, 1994). In addition, cognitive symptoms are affected in schizophrenics demonstrated by a reduced working memory and attention (Abi-Dargham et al., 2000).

1.3.2.1 Abnormalities in schizophrenia

Numerous brain abnormalities in schizophrenics have been reported. Some alterations include enlargement of ventricular system (Hyde and Weinberger, 1990) and basal ganglia (McCarley et al., 1999) as well as prominent sulci in the cortex (Rieder et al., 1979). In addition, decreased amygdala-hippocampal size (Hulshoff Pol et al., 2004) and cerebral volume (Wright et al., 2000) have been observed.

Genetic studies have identified numerous candidate genes implicated in schizophrenia: neuregulin-1 (NRG1; (Stefansson et al., 2002)), dysbindin (DTNBP1; (Straub et al., 2002)), G72 (Chumakov et al., 2002), D-amino acid oxidase (DAAO; (Chumakov et al., 2002)), RGS4 (Mirnics et al., 2001), catechol-O-methyltransferase (COMT; (Akil et al., 2003)) and proline dehydrogenase (PRODH; (Hoogendoorn et al., 2004)). Importantly, COMT is an enzyme involved in cortical DA metabolism, implicating the dopaminergic system in schizophrenia (Harrison and Owen, 2003)

Multiple theories have been proposed to understand the pathology behind schizophrenia. Hypofunction of N-methyl-D-aspartate (NMDA) receptors is one theory behind pathophysiology of schizophrenia that was developed upon the observation that NMDA antagonists (e.g. phencyclidine) lead to development of positive, negative and cognitive schizophrenia-like symptoms (Miyamoto et al., 2005). Another theory implicates norepinephrine, levels of which are elevated in the blood of schizophrenics (Breier et al., 1990). The serotonin system is also implicated since partial agonist improve negative symptoms in schizophrenics (Table I-IV) (Miyamoto et al., 2005), and alterations in this system have also been observed (Hashimoto et al., 1993). Finally, the most established theory behind schizophrenia is hyperactivity of the dopaminergic system (Bachus and Kleinman, 1996).

1.3.2.2 Dopamine-D2 and 5-HT1A receptors in schizophrenia

The genetics and neurochemistry of schizophrenia suggest a biological basis for the disease. Three basic classes of antipsychotics are used to treat schizophrenia (typical, atypical and dopamine partial agonists) all of which act on the dopamine systems. In addition, many of the presently used antipsychotics have affinities for other proteins, transporters and GPCRs such as 5-HT1A receptors (Table I-II). Interestingly, to date, all the drugs which demonstrate antipsychotic efficacy have some affinity for dopamine-D2 receptors (Miyamoto et al., 2005) and psychosis can be induced by dopamine enhancing drugs (Harrison, 2000). Dopamine plays a pivotal role in mediating schizophrenic behaviour (Noble, 2000) and dysregulation of the dopamine system has been implicated in schizophrenia. Furthermore, antipsychotics that block dopaminergic neurotransmission

Table I-IV. Mechanism of actions of antipsychotics and their putative clinical efficacy and consequences.

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Mechanisms	*Potential clinical efficacy*	*Potential consequences*
D_2R antagonism	↓ positive symptoms	EPS ↑Negative symptoms ↑Cognitive symptoms
D_2R partial agonism	↓ positive symptoms ↓ negative symptoms ↓ cognitive symptoms	Little or no EPS Behavioral activation
↑DA and NE release in the PFC	↓ negative symptoms ↓ cognitive symptoms ↓ depressive symptoms	Behavioral activation
ACh release in the PFC	↓ cognitive symptoms	
$5\text{-}HT_{2A}$ antagonism	↓ negative symptoms	↓ EPS
$5\text{-}HT_{1A}$ partial agonism	↓ negative symptoms ↓ cognitive symptoms ↓ anxiety symptoms ↓ depressive symptoms	
Muscarinic R antagonism	↓ EPS	↑ Anticholinergic symptoms e.g. dry mouth, constipation tachycardia
Muscarinic R agonism	↓ psychotic symptoms ↓ cognitive symptoms	
Glutamate modulation	↓ positive symptoms ↓ negative symptoms ↓ cognitive symptoms ↓ illness progression	

NE, norepinephrine; Ach, acetylcholine; PFC, prefrontal cortex; EPS, extrapyramidal symptoms.

by inhibiting DRD2 produce the best response rates in schizophrenics (Missale et al., 1998; Lewis and Levitt, 2002). Overexpression of key regulators of dopaminergic neurotransmission, such as DRD2, may be in part responsible for the elevated dopamine levels observed in schizophrenic patients (Abi-Dargham et al., 2000; Seeman and Kapur, 2000; Gainetdinov et al., 2001).

Binding studies in post-mortem brain tissue and imaging studies indicate that, in schizophrenia, DRD2 receptors are upregulated in the basal forebrain, resulting in increased dopaminergic neurotransmission (Abi-Dargham et al., 2000; Seeman and Kapur, 2000; Gainetdinov et al., 2001). A number of polymorphisms located in the *DRD2* were shown to associate with schizophrenia (Arinami et al., 1997; Golimbet et al., 2003) and altered DRD2 expression (Pohjalainen et al., 1998; Jonsson et al., 1999). One functional promoter polymorphism (-141C Ins/Del) with low promoter activity in the presence of -141C Del genotype is negatively associated with schizophrenia (Arinami et al., 1997; Ohara et al., 1998).

Another receptor altered in schizophrenia is the 5-HT1A receptor. The density of 5-HT1A receptor is found to be elevated in the dorsolateral prefrontal cortex and anterior cingulate gyrus in schizophrenics (Hashimoto et al., 1991; Hashimoto et al., 1993; Burnet et al., 1996). On the other hand, in the amygdala, 5-HT1A receptor levels were decreased in affected individuals (Yasuno et al., 2004). In addition, some antipsychotics activate 5-HT1A receptors (Li et al., 2004; Newman-Tancredi et al., 2005) and 5-HT1A agonists enhance cognitive performance in schizophrenics (Sumiyoshi et al., 2001a). Genetic analysis of the 5-HT1A receptor polymorphism C(-1019)G revealed an association with

schizophrenia (Huang et al., 2004). Therefore, altered transcriptional regulation of *DRD2* and *HTR1A* could account for the disease state and the observed symptoms.

1.3.3 Mental Retardation

Mental retardation (MR) affects up to 2% of the population and is attributed to environmental and genetic factors (Chechlacz and Gleeson, 2003; Raymond and Tarpey, 2006; Ropers, 2006). The genetics of MR demonstrate recessive, dominant, autosomal and X-linked inheritance modes (Raymond, 2006; Ropers, 2006). Diagnosis of MR is based on criteria which include impairments in intellectual function, self-care and social/interpersonal skills. The onset of this disability takes place prior to the age of 18 (Branchi et al., 2003). Mental Retardation in the absence of recognizable neurological or physical deficits is characterized as Non-Syndromic Mental Retardation (NSMR) which is inadequately understood and represents the most common form of MR (Raymond and Tarpey, 2006; Ropers, 2006; Rogaeva et al., 2007a).

Environmental factors predisposing to MR can include *in utero* alcohol exposure, infectious diseases, undernourishment at the time of pregnancy, premature delivery and injury (Chechlacz and Gleeson, 2003). Approximately 5% of the affected individuals demonstrate hereditary predisposition to MR and 30% are due to early alterations in embryonic development as a result of chromosomal changes or prenatal damage. MR can be subdivided into four groups: mild (IQ 50-55 to ~70), moderate (IQ 35-40 to 50-55), severe (IQ 20-25 to 35-40), and profound (IQ 20 or 25). Individuals with mild mental retardation constitute about 85% of the affected individuals and moderate MR is found in 10%, severe MR in 3-4% and profound MR is found in 1-2% of individuals with MR.

MR is more prevalent in males with 1.5:1 ratio of males to females affected (American Psychiatric Association, 1994). Consequently, inheritance of MR is commonly X-linked (XLMR) and represents 5% of total diagnosed MR individuals (Branchi et al., 2003).

1.3.3.1 Genes implicated in Mental Retardation

A great deal of research has focused on trying to identify genes implicated in MR. A number of genes with mutations (e.g. p21-Activated Kinase 3 (*PAK3*; (Verot et al., 2003)), Protease, Serine, 12 (*PRSS12*; (Molinari et al., 2002)), Cereblon (Higgins et al., 2004) and GDP Dissociation Inhibitor 1 (*Gdi1*; (D'Adamo et al., 2002))) have been implicated in NSMR (Raymond and Tarpey, 2006). The complexity of this disorder is highlighted by the fact that mutations in the same gene (e.g. Aristaless Related Homeobox (ARX)) are associated with both NSMR and MR (Raymond and Tarpey, 2006; Rogaeva et al., 2007a).

Recently, a deletion mutation in the *Freud-1/CC2D1A* was linked to NSMR (Basel-Vanagaite et al., 2003; Basel-Vanagaite et al., 2006; Raymond and Tarpey, 2006). Initial analysis detected an autosomal recessive NSMR locus on 19p13.12-p13.2 (Basel-Vanagaite et al., 2003), which was then mapped to a ~900-kb region with a 3.6-kb deletion in the *CC2D1A*. This deletion removes a region included within introns 13 to 16 (16/64; IVS13_IVS16del) and introduces a frame-shift mutation resulting in a premature stop codon after translation of 30 nonsense amino acids (G408fsX437) (hFreud-1_L NSMR, Figure I-17) (Basel-Vanagaite et al., 2006; Basel-Vanagaite et al., 2007). Importantly, in lymphoblasts of affected individuals, truncated Freud-1 protein was expressed, suggesting that the remainder of the protein could have dominant negative

qualities by recruiting co-repressor proteins (Basel-Vanagaite et al., 2006; Rogaeva et al., 2007a).

The possible involvement of Freud-1 in long term memory formation is also supported by its positive effect on NF-κB activity (Matsuda et al., 2003). Basel-Vanagaite *et al.*, with help of immunoprecipitation, do not observe direct interaction between Freud-1 and p65 or p50 subunits of NF-κB *in vivo* (Basel-Vanagaite et al., 2006). It is nonetheless evident that NF-κB is implicated in synaptic plasticity and transmission (Albensi and Mattson, 2000; Freudenthal et al., 2005; Kaltschmidt et al., 2006; O'Riordan et al., 2006) which might be partially dependent on Freud-1 activity even if no direct interaction has been reported.

Serotonin 1A receptors have been implicated in regulating cognitive function. Selective 5-HT1A receptor antagonists improve cognitive performance (Sumiyoshi and Meltzer, 2004; Schechter et al., 2005); however, 5-HT1A KO mice exhibit impaired intellectual ability (Sarnyai et al., 2000). Another GPCR gene, the *DRD2*, is also implicated in establishing a healthy mental state. The striatal-cortical loops are implicated in learning and cognition (Graybiel, 2005) and a mouse model with overexpressed *DRD2* in striatum has a reduced working memory that is irreversible, revealing a developmental role of the dopamine-D2 receptor in memory formation (Kellendonk et al., 2006; Rogaeva et al., 2007a). Furthermore, DRD2 is thought to play a major role in cognitive performance. A number of lines of evidence link the dopaminergic system with cognitive processing. Patients affected by Parkinson's disease demonstrate reduced short-term and working memory (Cooper et al., 1993) and the cerebral ageing accompanied by dopaminergic insufficiency with symptoms such as reduced reasoning and problem

solving ability are improved by dopaminergic agonists (Ollat, 1992). Furthermore, a polymorphism in the *DRD2* (Taq1A; rs#1800497) is associated with deficit in visuospatial ability (Berman and Noble, 1995). Finally, the dopaminergic system is implicated in cognitive skills (e.g. working memory, abstract reasoning and cognitive flexibility) (Previc, 1999). Although one study failed to detect an association with intelligence (Moises et al., 2001) substantial evidence implicates *DRD2* in memory and cognitive function.

Understanding this heterogeneous disorder and the roles of such proteins as Freud-1, 5-HT1A, DRD2 and NF-κB in its regulation, will be essential to correcting defects in cognitive development that lead to mental retardation.

1.4 RATIONALE, GOALS AND OBJECTIVES

Imbalances in the transcriptional regulation of key genes implicated in dopaminergic and serotonergic neurotransmission, such as *DRD2* and *HTR1A*, may be in part responsible for predisposition to psychiatric disorders. The abnormal expression levels of *DRD2* and *HTR1A* is implicated in depression (Larisch et al., 1997; Li et al., 1999; Osterlund et al., 1999; Koks et al., 2006; Abbas et al., 2007), schizophrenia (Hashimoto et al., 1991; Hashimoto et al., 1993; Burnet et al., 1996; Arinami et al., 1997; Missale et al., 1998; Lewis and Levitt, 2002; Golimbet et al., 2003) and mental retardation (Berman and Noble, 1995; Sarnyai et al., 2000; Graybiel, 2005; Kellendonk et al., 2006). In addition, a repressor of *HTR1A* expression, *Freud-1* (Ou et al., 2000; Ou et al., 2003; Lemonde et al., 2004), and its linkage with NSMR (Basel-Vanagaite et al., 2003; Basel-Vanagaite et al., 2006), implicates dys-regulated transcriptional control in development of this disorder. The *in vivo* immunohistochemical analysis of Freud-1 distribution revealed its localization to dopamine-D2 and 5-HT1A receptor positive neurons (Ou et al., 2003). These observations have lead to the hypothesis that transcriptional regulation of these genes may be regulated by Freud-1. It is also important to understand the mechanisms involved in homeostatic regulation of gene expression and how it is altered in affected patients, in order to define new, more effective and safer drug targets.

The goal of this project is to further understand transcriptional regulation of *DRD2* and *HTR1A*. The hypothesis for this thesis is that the Freud-1 transcriptional repressor is a regulator of *DRD2* and *HTR1A* at a novel polymorphic element and previously characterized repressor element, respectively. The objectives are to show that

1) the long isoform of Freud-1 is a regulator of the *HTR1A*; 2) Freud-1 is a regulator of *DRD2* at a functional polymorphic element; 3) selected polymorphisms in the *DRD2* are associated schizophrenia and MDD.

In this thesis I present evidence supporting the main hypothesis. Specifically, in chapter II we demonstrate that the long isoform of Freud-1 is a repressor of the *HTR1A* and is endogenously bound to the promoter. In the chapter III we describe a novel polymorphic repressor element in the *DRD2* and demonstrate Freud-1 as a functional repressor at this element. Finally, in chapter IV we present an association study of two previously unexamined polymorphisms and the Taq1A polymorphism with MDD and schizophrenia. The question still remains as to what other genes may be regulated by Freud-1, and which functions of Freud-1 contribute to the symptoms of NSMR.

CHAPTER II - THE MENTAL RETARDATION GENE FREUD-1/CC2D1A ENCODES A LONG ISOFORM THAT BINDS CONSERVED DNA ELEMENTS TO REPRESS GENE TRANSCRIPTION

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ABBREVIATIONS

CHIP, chromatin immunoprecipitation; CRM1, chromosome region maintenance 1; DM14, *Drosophila melanogaster* 14 domain; DRE, dual repressor element; EMSA, Electrophoretic Mobility Gel Shift Assay; Freud-1, Five prime Repressor Under Dual repression binding protein 1; Freud-1_L, long isoform of Freud-1; Freud-1_S, short isoform of Freud-1; GFP, Green Fluorescent Protein; LMB, Leptomycin B; NF-κB, Nuclear

Factor-kappaB; QPCR, quantitative PCR; STAT2, signal transducer and activator or transcription 2; SUMO, small ubiquitin-like modifier.

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Authors' contributions: I have performed most of the work depicted in this manuscript including the writing of the preliminary manuscript. Dr. Paul Albert and I have edited the follow up versions and prepared it for submission.

2.1 ABSTRACT

The *CC2D1A/Freud-1* gene has recently been linked to non-syndromic mental retardation, and a short isoform of mouse Freud-1 protein can repress the 5-HT1A receptor gene in rodent cells. In this study, we addressed the expression, localization, and regulation of the human 5-HT1A receptor gene by an uncharacterized long isoform of human Freud-1 protein (Freud-1_L). We show that human CC2D1A/Freud-1 RNA is expressed in brain and peripheral tissues and encodes short and long isoforms, which differ by an upstream in-frame translational start site. While previous studies identified the short isoform of Freud-1 as the predominant isoform in rodent cells, we demonstrate that the long isoform is more abundant in human cells, especially in the nuclear fraction. The nuclear localization of Freud-1_L was enriched upon inhibition of CRM1/exportin 1-dependent nuclear export, indicating a dynamic regulation of Freud-1 nuclear localization. Consistent with a functional role in the nucleus, human Freud-1_L bound specifically to its dual repressor element in the 5-HT1A receptor gene *in vitro* and repressed transcription from these sites. Importantly, chromatin immunoprecipitation using antibodies specific for human Freud-1_L demonstrated that it is bound to these dual repressor elements in chromatin, indicating a functional role in regulating the basal expression of the 5-HT1A receptor gene. Taken together, these results indicate that Freud-1_L is the major isoform that regulates the human 5-HT1A receptor gene. Disruption of transcriptional regulation by Freud-1 may play a role in abnormal brain function leading to mental retardation.

2.2 INTRODUCTION

The molecular basis for mental retardation involves multiple genes and mutations in transcriptional regulators have been linked genetically to developmental disorders, such as Rett or Rubinstein-Taybi syndromes (Alarcon et al., 2004; Caballero and Hendrich, 2005; Hong et al., 2005; Roelfsema et al., 2005). Recently, a gene on chromosome 19p13.12-p13.2, denoted coiled-coil and C2 domain containing 1A (*CC2D1A*) or Five prime Repressor Under Dual repression binding protein 1 (*Freud-1*), was linked to non-syndromic mental retardation (Basel-Vanagaite et al., 2003; Basel-Vanagaite et al., 2006). In affected individuals, a 3.6-kb deletion in the *CC2D1A* gene was identified. The deletion introduces a frame shift mutation resulting in expression of a truncated protein (Basel-Vanagaite et al., 2006).

The Freud-1 protein contains conserved *Drosophila melanogaster* 14 domains (DM14) of unknown function, a helix-loop-helix (HLH) domain and a protein kinase C conserved region 2 domain (C2) (Figure II-1*A*) (Ou et al., 2003; Basel-Vanagaite et al., 2006). The mutation linked with non-syndromic mental retardation encodes a protein that lacks one C-terminal DM14, C2 and HLH domains, which is predicted to abolish DNA binding and repressor activity of Freud-1 (Figure II-1*B*) (Ou et al., 2003; Albert and Lemonde, 2004; Rogaeva et al., 2007a). In addition, both long (Freud-1_L) and short (Freud-1_S) Freud-1 isoforms have been identified. They differ by an upstream in-frame translational start site, encoding an additional 348 amino acids at the amino-terminus (Figure II-1*A*) (Matsuda et al., 2003; Ou et al., 2003; Basel-Vanagaite et al., 2006). Database analysis revealed a human Freud-1_S (GenBank accession no. AK023399) and mouse Freud-1_L proteins (GenBank accession no. BC016188) (Figure II-1*B*). Mouse

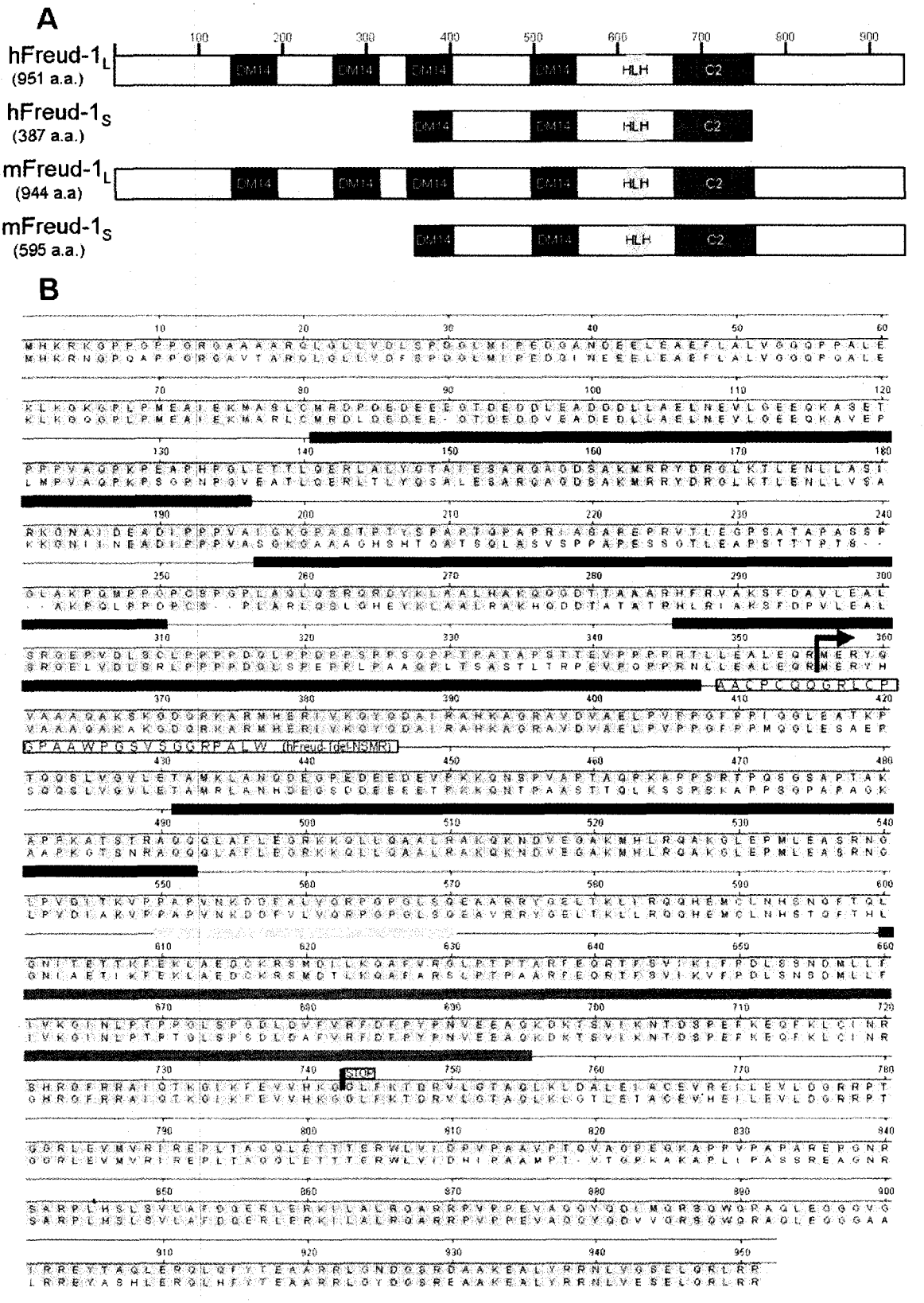

Figure II-1. Alignment of human and mouse Freud-1 isoforms.

(**A**) Schematic representation of human Freud-1_L (**hFreud-1_L**; AB097002) and potential short isoform (**hFreud-1_S**; AK023399) similar to mouse Freud-1_S. Mouse Freud-1_L (**mFreud-1_L**; BC016188) and Freud-1_S (**mFreud-1_S**; ABC56419) isoforms are also shown. Shaded boxes represent conserved domains: DM14 (**black**), HLH (**light gray**), and C2 domains (**dark gray**). Amino acid position is shown above human Freud-1_L sequence with their total number depicted below each protein. (**B**) Protein alignment of human (top) and mouse (bottom) Freud-1_L. Conserved amino acids are shaded in gray with domains highlighted in the similar color scheme as in panel A (**black**, DM14; **light gray**, HLH; **dark gray**, C2). **Arrow** indicates the first methionine of mouse and human Freud-1_S proteins and **STOP** demonstrates premature stop codon in human Freud-1_S. Nonsense amino acid sequence and a premature stop codon of human Freud-1 protein (**hFreud-1del-NSMR**) identified in patients with non-syndromic mental retardation is boxed above the deleted sequence.

Freud-1_S, lacks the first two DM14 domains, and was shown to be a transcriptional repressor of the serotonin-1A (5-HT1A) receptor gene with activity at the conjoined dual repressor elements (5'DRE and 3'DRE) in the promoter of the 5-HT1A receptor gene (Ou et al., 2003; Lemonde et al., 2004). However, the functional activity of the human Freud-1_L isoform has not been addressed.

Freud-1 is widely expressed throughout the rat brain, including in cortex, hippocampus and raphe nuclei, suggesting a global regulatory function. In addition, Freud-1 is co-expressed with 5-HT1A receptors, consistent with a role in regulating serotonergic function (Ou et al., 2003). Freud-1 RNA is also detected in mouse brain at embryonic day 12, suggesting a possible role in brain development (Basel-Vanagaite et al., 2006). However, little is known of the function of the human *CC2D1A/Freud-1* gene. Previous work has shown that deletion of the DRE sites in the rat or human 5-HT1A promoter results in strong upregulation of its transcriptional activity (Lemonde et al., 2004). Furthermore, regulation of rat 5-HT1A receptor expression by Freud-1 was demonstrated by depleting endogenous Freud-1 with an antisense construct, consequently upregulating 5-HT1A receptor expression (Ou et al., 2003). However, these studies did not address which endogenous Freud-1 isoform is involved. Here, we provide evidence that human Freud-1_L is a critical transcriptional repressor of the human 5-HT1A receptor gene.

2.3 MATERIALS AND METHODS

Plasmid construction

A plasmid containing human Freud-1_L cDNA (GenBank accession no. AB097002) was obtained from Dr. Matsuda, ASAH1 KASEI Corporation, Shizuoka, Japan (Matsuda et al., 2003), amplified by PCR and subcloned into EcoRI-cut pTriEX4 (Novagen, Madison, WI, USA) to generate both sense and antisense constructs. The human Freud-1_L cDNA was also subcloned into pcDNA3 (Invitrogen, Burlington, ON, CA) and pEGFP N3 vectors (Clontech, Mountain View, CA, USA). The stop codon of human Freud-1_L was mutated using QuikChange™ XL Site-Directed Mutagenesis (Stratagene, Cedar Creek, TX, USA) for in-frame incorporation of a carboxy-terminal His-tag and Green Fluorescent Protein (GFP) to generate human Freud-1_L-GFP. Reporter constructs were previously described (Lemonde et al., 2004). Briefly, human 5-HT1A DRE sequences (h5-HT1A DRE; Table II-I) were cloned upstream of the SV40 promoter into pGL3-Promoter vector (Promega, Madison, WI, USA) either individually (h5-HT1A 3'DRE and h5-HT1A 5'DRE) or joint (h5HT1A 5'&3'DRE).

Expression and purification of human Freud-1_L and subcellular fractionation

Escherichia Coli BL21 (DE3) (Novagen, Madison, WI, USA) was transformed with human Freud-1_L expression plasmid, grown overnight and induced at OD_{600}=0.6 with 1 mM isopropyl-β-D-thiogalactopyranoside (Wisent, St-Bruno, QC, CA) for 3 h at 37°C. Human Freud-1_L was purified under native conditions using Ni-NTA beads (Qiagen, Mississauga, ON, CA) and dialyzed against DNA binding buffer (20 mM HEPES, 0.2 mM EDTA, 0.2 mM EGTA, 100 mM KCl, 5% glycerol, pH 7.9) to facilitate protein-DNA interaction.

Table II-I. Sequences for dual repressor and control DNA elements.

Shown are the oligonucleotide sequences of dual repressor elements (DRE) in human (h) and rat (r) 5-HT1A receptor gene used for primers in EMSA and in generating reporter constructs. E2F is a non-specific competitor for EMSA.

Name	**Sequence**
r5-HT1A 5'DRE	5'-AGATGGCGCTCTGAAGCAATTGCCGGA
r5-HT1A 3'DRE	5'-AGGTGGCGGCATAAGCAAGCCCTTATTGCACAGAGC
r5-HT1A 3'&5'DRE	5'-AGATGGCGCTCTGAAGCAATTGCCGGAAGGTGGCGGCATAAGCAAGCCCTTATTGCACAGAGC
h5-HT1A 5'DRE	5'-AGATGGCACTCTAAAACATTTGCCAGA
h5-HT1A 3'DRE	5'-AGGTGGCGACATAAAACCTCATTGCTTAGAACT
h5-HT1A 3'&5'DRE	5'-AGATGGCACTCTAAAACATTTGCCAGAAGGTGGCGACATAAAACCTCATTGCTTAGAACT
E2F	5'-TATAGTGTACTCTACTATTCTGCTC

Subcellular fractionation was performed as previously described (Czesak et al., 2006). Briefly, cells were allowed to pre-swell for 10 min at 4°C in the extraction buffer lacking NP-40, DTT, spermidine, spermine, PMSF and Protease Inhibitors, following which pelleted cells were lysed for 10 min on ice with extraction buffer (10 mM KCl, 10 mM Na-HEPES, pH 7.6, 1.5 mM $MgCl_2$, 0.1% NP-40, 0.5 mM DTT, 0.5 mM spermidine, 0.15 mM spermine, 1 mM PMSF, 1x Protease Inhibitor Cocktail (Roche, Laval, QC, CA)). The lysate was centrifuged and the supernatant reserved as the non-nuclear fraction. The resultant nuclear pellet was collected and washed with buffer (50 mM NaCl, 20 mM Na-HEPES, pH 7.6, 25% glycerin, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM spermidine, 0.15 mM spermine, 1 mM PMSF, 1x Protease Inhibitor Cocktail). Finally, nuclear extraction buffer (500 mM NaCl, 20 mM Na-HEPES, pH 7.6, 25% glycerin, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM spermidine, 0.15 mM spermine, 1 mM PMSF, 1x Protease Inhibitor Cocktail) was used to lyse nuclei. Fractions were analyzed with Western blotting for cross contamination between nuclear and non-nuclear compartments and presence of Freud-1.

Cell culture, transient transfections, siRNA and luciferase reporter assays

Human embryonic kidney (HEK293), human neuroblastoma (SK-N-AS; provided by Dr. Michael Bannon, Wayne State University, Detroit, USA), human neuroblastoma (SK-N-SH; provided by Dr. Lakshmi Devi, New York University, N.Y., USA), mouse/rat neuroblastoma x glioma hybrid (NG108-15) and mouse embryonic fibroblast cells (NIH/3T3) were maintained in Dulbecco's Modified Eagle's Medium (Wisent, St-Bruno, QC, CA) supplemented with 10% fetal calf serum. A7 human melanoma cells (provided by Dr. John Hartwig, Brigham and Women's Hospital, Boston, MA (Ohta et al., 1999;

Lin et al., 2001)) were grown in Minimum Essential Medium (Wisent, St-Bruno, QC, CA) supplemented with 8% newborn calf serum, 2% fetal calf serum, and 500 μg/ml of G418 (Invitrogen, Burlington, ON, CA). All of the above cells were grown at 37°C in 5% CO_2 to 50-60% confluence, and the media replaced 12 h prior to transfection. Cells for nuclear/cytosolic trafficking analysis were treated with 10 ng/ml of Leptomycin B (LMB; Sigma), a chromosome region maintenance 1 (CRM1)/exportin 1 inhibitor, for 3 h as previously described (Nagita et al., 2003) or with equivalent volume of LMB solvent (70% methanol) as a control. Subcellular distribution of GFP-tagged human Freud-1_L (Freud-1_L-GFP) transfected HEK293 cells was visualized using Zeiss Axiophot II microscope and a Retiga Qimage CCD camera. The fluorescence was quantified with Adobe Photoshop version 5.0 in nuclear and cytosolic cellular compartments in the presence and absence of LMB using fixed size selection tool and a histogram.

The specificity of anti-hFreud-1_L antibody was validated by depleting endogenous Freud-1 in HEK293 cells with siRNA. The specific stealth siRNA (5'-ggcgcucuaucagacagcaauugaa-3') or a scrambled negative control siRNA**C** (5'-ggcucucuaagagacaacuugcgaa-3') designed to target hFreud-1_L (https://rnaidesigner.invitrogen.com/sirna/; Invitrogen, Burlington, ON, CA) were transfected with HiPerFect (Qiagen, Mississauga, ON, CA) into HEK293 cells. The final siRNA concentration was 20 nM and transfection efficiency was approximately 90% as assessed by BLOCK-iT™ Fluorescent Oligo (Invitrogen, Burlington, ON, CA). The downregulation of Freud-1 was analyzed by Western blotting with anti-hFreud-1_L antibody following 72 h treatment with siRNA.

HEK293, NIH/3T3 and A7 cells were transfected with Lipofectamine and Plus reagent (Invitrogen, Burlington, ON, CA) using total 1.1 μg of DNA:1 μl of Lipofectamine:1 μl of Plus reagent. For reporter assays, 0.5 μg of reporter constructs were co-transfected with 0.1 μg of pCMV-βgal (Clontech, Mountain View, CA, USA) and 0.5 μg of human Freud-1_L-expressing plasmid or empty vector. For luciferase reporter assays, cells were lysed with 200 μl of 1x Reporter Lysis Buffer (Promega, Madison, WI, USA) and activity of supernatants assessed using a 1250 luminometer (Bio Orbit, Turku, Finland). The β-galactosidase activity was assayed using 4-methyl-umbelliferyl-β-D galactoside as substrate (Sigma) and product quantified using a Perkin Elmer LS50 spectrofluorometer (λex=350 nm, λem=450 nm). The ratio of luciferase/β-galactosidase activity was determined in triplicate samples, and normalized to pGL3-Promoter vector (Promega, Madison, WI, USA).

Electrophoretic Mobility Gel Shift Assay (EMSA)

Complementary oligonucleotides (IDT, Coralville, IA, USA) for rat (r5-HT1A 5'DRE and r5-HT1A 3'DRE) and human 5-HT1A DREs (h5-HT1A 3'DRE and h5-HT1A 5'DRE) (Table II-I) were annealed and end-labeled with [α-^{32}P] dCTP using 2.5U Klenow (New England Biolabs, Pickering, ON, CA) and purified over Sephadex G-50 column (GE HealthCare, Baie d'Urfe, QC, CA) as previously described (Lemonde et al., 2004). Two μg/sample of bacterially expressed, purified and dialyzed human Freud-1_L was preincubated with or without competitor DNA: (r5-HT1A 5'DRE, r5-HT1A 3'DRE, r5-HT1A 3'&5'DRE, h5-HT1A 3'DRE, h5-HT1A 5'DRE and E2F; Table II-I) or 1 μl of anti-His antibody (Covance, Berkeley, CA, USA) in a 25 μl reaction containing DNA binding buffer (20 mM HEPES, 0.2 mM EDTA, 0.2 mM EGTA, 100 mM KCl, 5%

glycerol and 2 mM DTT, pH 7.9) and 250 ng of Herring sperm ssDNA and incubated at 25°C for 30 min. Double-stranded ^{32}P-labeled probe (50,000 cpm/sample) was then added and incubated for an additional 20 min at room temperature. The DNA/protein complexes were separated on a nondenaturing 5% polyacrylamide gel at 4°C, dried and examined by autoradiography (Ou et al., 2000).

Antibodies, Western and Northern Blot Analyses

Three anti-Freud-1 antibodies were used: anti-N-mFreud-1$_S$, raised to an N-terminal peptide of mouse Freud-1$_S$ (ETPKKHNTPAASTTQLK; (Ou et al., 2003)); anti-hFreud-1$_L$, generated against bacterially-expressed and purified (Ni-NTA beads; Qiagen, Mississauga, ON, CA) S/His-tagged human Freud-1$_L$ (Cedarlane, Hornby, ON, CA); and anti-CC2D1A, targeting N-terminal of human Freud-1$_L$ (amino acid 1-50; Bethyl Laboratories Inc., Montgomery, TX, USA) (Figure V-1). For Western blot analysis, PVDF membranes were incubated with anti-N-mFreud-1$_S$ (1:500), anti-hFreud-1$_L$ (1:20,000), anti-cRaf (1:3,000; BD Biosciences, Mississauga, ON, CA) and anti-Histone H1 (1:1,000; Upstate Biotechnology, Lake Placid, NY, USA) overnight at 4°C followed by horseradish peroxidase-linked anti-rabbit (1:2,000; New England Biolabs, Pickering, ON, CA) or anti-mouse (1:2,000; Jackson Immunoresearch Laboratories, West Grove, PA, USA) secondary antibody and tertiary BM chemiluminescence blotting substrate (Roche, Laval, QC, CA).

For Northern blot analysis, human Freud-1$_L$ cDNA was digested with XcmI to generate a 444-bp probe specific to the 5'-portion of human Freud-1$_L$ (nucleic acid 463-919, GenBank accession no. AB097002). The probe was gel-purified, labeled using [α-^{32}P] dATP as per Strip-EZ™ Protocol (Ambion, Austin, TX, USA) and hybridized in

ULTRAhyb™ (Ambion, Austin, TX, USA) overnight to Northern blots (Clontech, Mountain View, CA, USA) followed by extensive washing as per Strip-EZ™ protocol (Ambion, Austin, TX, USA). The blots were stripped and re-probed with radioactively labeled β-actin cDNA (Clontech, Mountain View, CA, USA). The resultant blots were visualized by autoradiography.

Chromatin Immunoprecipitation (CHIP)

CHIP assays were performed as per manufacturer's protocol (Upstate Biochemical, Charlottesville, VA, USA). Briefly, cells were washed three times with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4), crosslinked for 15 min at 25°C with PBS supplemented with 1 mM $MgCl_2$ and 1% formaldehyde (v/v). Fixed cells were rinsed again three times with PBS and lysed (Nowak et al., 2005). Crosslinked genomic DNA was sheared using 60 Sonic Dismembrator (Fisher, Ottawa, ON, CA) 15x at setting 7 for 20 s with 15 s intervals. Addition of 212- to 300-µm diameter glass beads (Sigma) (Weinmann et al., 2001) ensured optimal shearing as verified by agarose gel (100-400-bp fragments; data not shown). Sheared lysates were immunoprecipitated overnight at 4°C with anti-hFreud-1_L (1:1,000) or anti-CC2D1A (1:1,000) antibodies. De-crosslinking was done overnight at 65°C, followed by 1 h digestion with proteinase K (Sigma) and phenol/chloroform extraction. Results were analyzed using quantitative PCR (QPCR) with primers designed to amplify 151-bp region containing 5-HT1A 5'&3'DRE (5'-ctgtcttcctctttctaaaaagttgttg-3' and 5'-ccggctagcctctcttaatctcag-3'). The amplification cycles were: 92°C for 10 min; 92°C for 30 s; 54°C for 30 s; 72°C for 30 s; 84°C for 20 s (25 cycles), and terminated at 72°C for 10 min using (Rotor-Gene RG-3000; Corbett Life Science, Sydney, Australia) and quantified using SYBR green (Molecular Probes,

Eugene, OR, USA) incorporation following amplification with Qtaq™ DNA polymerase mix (Clontech, Mountain View, CA, USA). The PCR products were further verified on an agarose gel (data not shown).

2.4 RESULTS

Tissue distribution of human Freud-1 RNA

The expression pattern of human Freud-1 RNA was examined by Northern blot analysis using a cDNA probe specific for the 5'-coding sequence of human Freud-1_L. The largest predicted size of Freud-1 transcript from GenBank is 3.6-kb (accession no. BC064981). However, results of our Northern blot analyses reveal three transcripts all of which are larger then the predicted transcript. We demonstrate two transcripts of human Freud-1 (4-kb and 5-kb) in all analyzed brain regions. In contrast, only the 4-kb RNA species was detected in peripheral tissues except for skeletal muscle in which a third transcript (6-kb) was present (Figure II-2; lane 3). Our results demonstrate that human Freud-1 RNA was expressed in all analyzed tissues with the highest expression in skeletal muscle (Figure II-2; lane 3). In the brain, Freud-1 was ubiquitously expressed with the highest expression in the cortex, cerebellum and putamen. Thus, human Freud-1 RNA is expressed as at least three major transcripts, which could encode for both Freud-1_L and Freud-1_S.

Expression and subcellular distribution of Freud-1 isoforms

The expression of Freud-1 protein was examined human (HEK293, SK-N-SH) or rodent (NG108-15) cell lines. Based on cDNA sequences reported in GenBank, CC2D1A/Freud-1 clones have predicted molecular mass of 67-kDa (mouse Freud-1_S), 43-kDa (human Freud-1_S), and 104-kDa (human and mouse Freud-1_L) (Figure II-1*A*). We used two different polyclonal antibodies with distinct specificities to Freud-1 isoforms. First, an antibody raised against an N-terminal mouse Freud-1_S (anti-N-mFreud-1_S) that detects rodent Freud-1_S but not Freud-1_L was used (Ou et al., 2003). Anti-N-mFreud-1_S antibody identified only a ~67-kDa species in human HEK293 cells (Figure II-3*A*). Selective

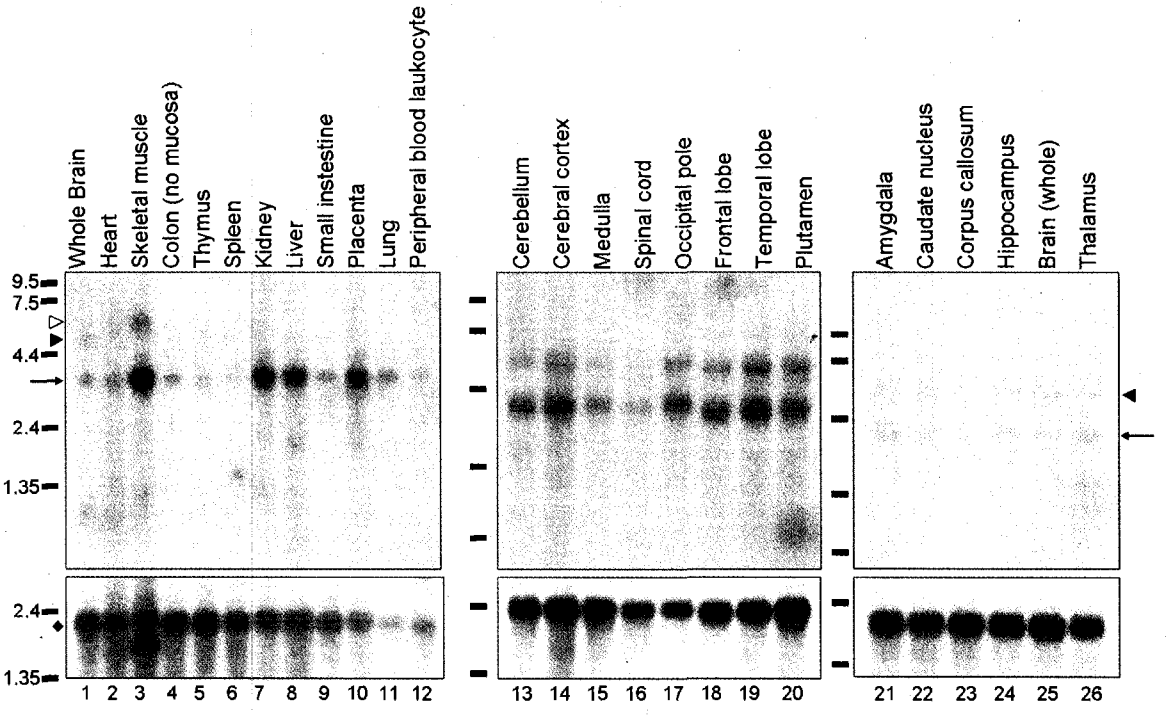

Figure II-2. Freud-1 RNA expression in adult human tissues.

Top panels are Northern blots of poly A^+ RNAs (Clontech) hybridized with a 5'-probe of human Freud-1_L cDNA. Bottom panels are re-probed blots with β-actin probe as a loading control. The RNA species for Freud-1 (**arrow**, and **open** and **closed arrowheads**) in addition to β-actin (**diamond**) are indicated. The molecular size standards (kb) are as shown.

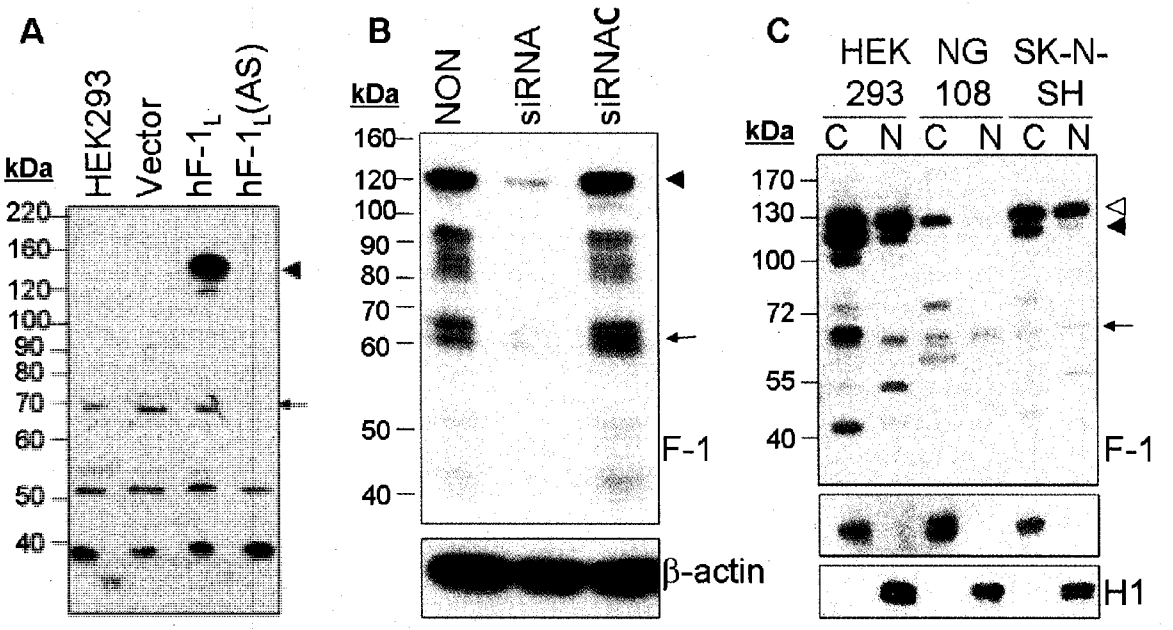

Figure II-3. Expression and subcellular localization of human Freud-1 isoforms.

(**A**) Detection of human Freud-1 isoforms in HEK293 cells with anti-N-mFreud-1_S antibody. HEK293 cells were non-transfected or transfected with vector, S/His-tagged human Freud-1_L (**hF-1_L**), or human Freud-1_L antisense (**hF-1_L(AS)**) plasmids. Whole cell lysates (50 μg) were examined by Western blotting, and overexpressed human Freud-1_L (**arrowhead**) and endogenous Freud-1_S (67-kDa; **arrow**) were visualized using anti-N-mFreud-1_S antibody. (**B**) Specificity of anti-hFreud-1_L antibody. HEK293 cells were treated with specific siRNA (**siRNA**), not treated (**NON**) or treated with a scrambled siRNA control (**siRNAC**). Western blot using anti-hFreud-1_L antibody was performed on the whole cell lysates. Equal loading was validated with anti-β-actin antibody. Note that all immunoreacted bands were depleted following siRNA treatment, but not siRNA**C**, indicating that these species are all derived from CC2D1A/Freud-1 gene. (**C**) Subcellular distribution of human Freud-1 isoforms. Nuclear (**N**; histone H1 positive) and cytosolic (**C**; c-Raf positive) fractions from HEK293, NG108-15 (**NG108**) and SK-N-SH cells

were analyzed using anti-hFreud-1_L (**F-1**), anti-c-Raf (74-kDa) and anti-histone H1 (**H1**; 32/33-kDa) antibodies. Freud-1 immunoreacted as three major bands: 130-kDa (Freud-1_L; **open arrowhead**), 120-kDa (Freud-1_L; **closed arrowhead**) and 67-kDa (Freud-1_S; **arrow**).

depletion of the ~67-kDa species upon transfection of an antisense human Freud-1_L construct confirmed that it corresponds to human Freud-1_S (Figure II-3*A*; arrowhead). Although, anti-N-mFreud-1_S antibody failed to identify endogenous Freud-1_L, transiently transfected human Freud-1_L was detected (Figure II-3*A*; arrow). A second antibody was generated against full-length human Freud-1_L (anti-hFreud-1_L) and its specificity validated by depleting immunoreactive bands with specific siRNA compared with untreated and scrambled siRNA controls (Figure II-3*B*). This anti-hFreud-1_L antibody that detected three major protein variants (67-, 120- and 130-kDa) in HEK293, SK-N-SH and NG108-15 cells (Figure II-3*C*). The 67-kDa band corresponds to Freud-1_S (Figure II-3*C*; arrow), while the 120/130-kDa doublet corresponds to Freud-1_L (Figure II-3*C*; arrowhead). The two molecular sizes of Freud-1_L could be due to differential post-translational modification. Thus, the anti-hFreud-1_L antibody revealed expression of both isoforms, showing that Freud-1_L is the predominant form.

The subcellular localization of Freud-1 isoforms was determined to address which isoform has a nuclear localization that would support its function as a transcriptional regulator. Western blot analysis of human HEK293 and SK-N-SH cells with anti-hFreud-1_L antibody detected both isoforms of Freud-1 in non-nuclear (c-Raf-enriched) and nuclear (histone H1-enriched) fractions (Figure II-3*C*). Importantly, in human cells Freud-1_L was abundant in the nuclear fraction, while Freud-1_S was very weakly detected. The nuclear fraction of 5-HT1A-negative HEK293 cells was enriched with 120/130-kDa Freud-1_L doublet in contrast to distribution in the 5-HT1A-positive SK-N-SH cells in which primarily the 130-kDa isoform was present. By contrast, in rodent NG108-15 cells Freud-1_L (130-kDa) was detected only in the cytosolic fraction while Freud-1_S isoform

was present in nuclear and cytosolic fractions. The predominant localization of human Freud-1_L in the nuclear fraction supports its potential role as a transcriptional regulator.

The mechanisms regulating the nuclear localization of Freud-1_L were examined in HEK293 cells transiently transfected with a human Freud-1_L-GFP construct (Figure II-4*A*). Semi-quantitative analysis of subcellular fluorescence revealed 30% nuclear vs. 70% non-nuclear human Freud-1_L-GFP distribution (Figure II-4*B*), consistent with the distribution of endogenous Freud-1_L detected by Western blot analysis (Figure II-3*C*). Treatment of HEK293 cells with Leptomycin B (LMB), an inhibitor of chromosome region maintenance 1 (CRM1)/exportin 1-mediated nuclear export, induced a 40% increase in nuclear fluorescence ($P<0.0001$; Figure II-4*B*). Similarly, treatment of untransfected HEK293 cells with LMB also enhanced an accumulation of endogenous Freud-1_L in the nuclear fraction compared to control (Figure II-4*C*). These data indicate that nuclear/cytosolic trafficking of human Freud-1_L is regulated by CRM1/exportin 1-dependent nuclear export, consistent with its role as a transcription factor.

Function of human Freud-1_L

Given its expression in the brain and nuclear localization, we addressed the repressor activity of human Freud-1_L at the conserved 5-HT1A dual repressor elements (DREs). The effect of overexpressing human Freud-1_L on reporter activity of human 5-HT1A DRE-luciferase reporter constructs (5'DRE, 3'DRE, or 5'&3'DRE; Table II-I) was examined in 5-HT1A-negative mouse NIH/3T3 or human A7 melanoma cells (Figure II-5). The DRE-containing constructs alone exhibited 40-60% reduced activity compared to the pGL3-Promoter vector control (Figure II-5), due to endogenous Freud-1 activity (Lemonde et al., 2004). Overexpression of human Freud-1_L further reduced the

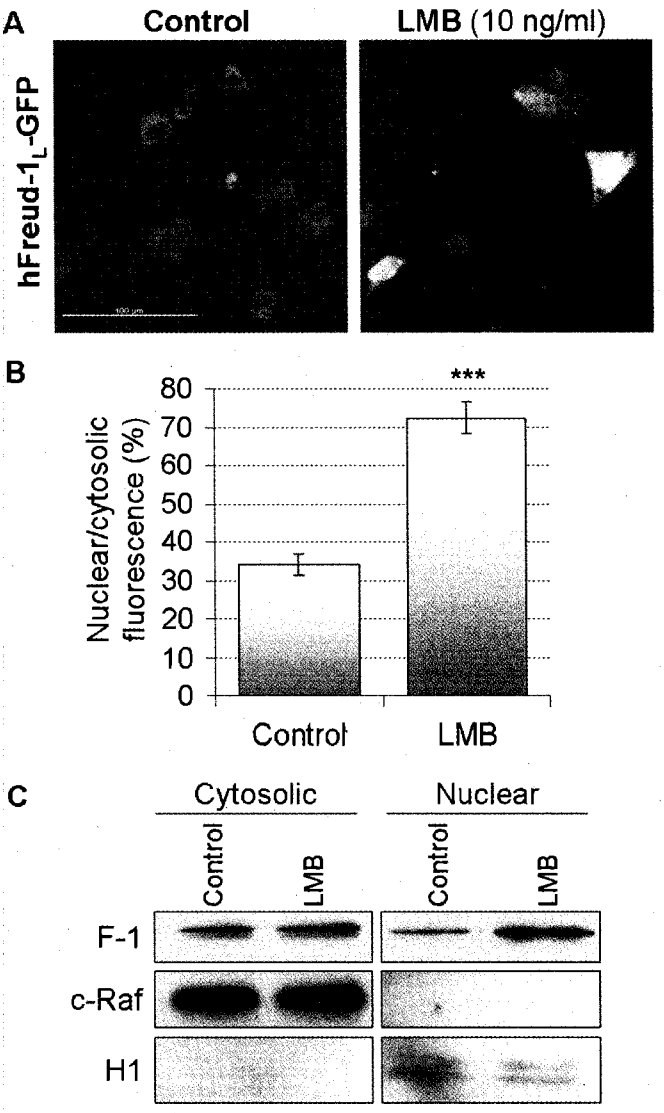

Figure II-4. Nuclear/cytosolic shuttling of hFreud-1_L isoform.

(**A**) Nuclear export of human Freud-1_L. HEK293 cells transfected with human Freud-1_L-GFP (**hFreud-1_L-GFP**) were either treated with CRM1/exportin 1 inhibitor (Leptomycin B; **LMB**) or not (Control). The GFP localization was monitored by fluorescence. Scale: 100 μm. (**B**) Subcellular fluorescence of human Freud-1_L-GFP. The data is represented as the ratio of nuclear to cytosolic fluorescence (%) as detected in twenty randomly-picked areas in cytoplasm and nucleus of healthy LMB treated and control cells. *** $P<0.0001$ (two-tailed unpaired t-test) (**C**) Nuclear export of endogenous Freud-1_L. Nuclear and

cytosolic fractions of HEK293 cells treated or not with LMB were analyzed by immunoblot using anti-hFreud-1_L (**F-1**), anti-c-Raf and anti-histone H1 (**H1**) antibodies. Freud-1_L protein was increased in the nuclear fraction of LMB-treated cells.

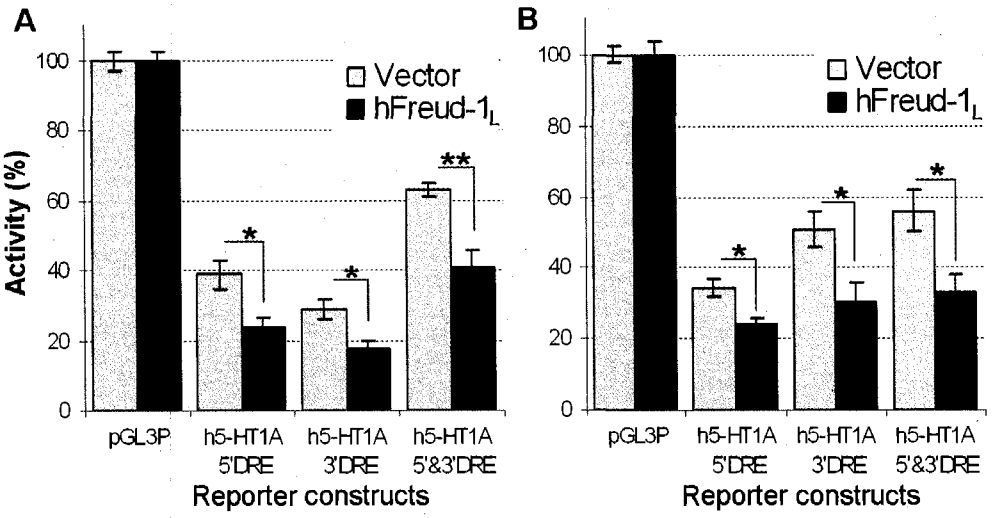

Figure II-5. Human Freud-1_L is a repressor at the human 5-HT1A dual repressor elements.

A7 (**A**) or NIH/3T3 (**B**) cells were transfected with reporter constructs containing the human 5-HT1A 5'DRE, 3'DRE and 5'&3'DRE (Table II-I). Promoter activity (%) normalized to transfection efficiency and empty pGL3-Promoter (**pGL3P**) vector was examined in the absence (vector control) or presence of human Freud-1_L expression construct (**hFreud-1_L**). DREs displayed basal repression of SV40 promoter activity that was enhanced by overexpression of human Freud-1_L (N≥3, ±S.E. *P<0.035, ** P=0.002 as analyzed by two-tailed unpaired t-test).

activity of the DRE-containing reporter constructs by 30-50% but did not alter activity of the pGL3- Promoter vector. Thus, human Freud-1_L displays repressor activity at the DREs of the human 5-HT1A receptor gene.

To address DNA binding activity of human Freud-1_L, Electrophoretic Mobility Gel Shift Assay (EMSA) was performed using purified recombinant His-tagged human Freud-1_L and radioactively labelled double-stranded DRE primers (Figure II-6*A*). Human Freud-1_L bound to both rat and human 5-HT1A DREs. The specificity of this interaction was demonstrated by the lack of protein-DNA complex in the absence of human Freud-1_L (data not shown); by supershift of the complex with anti-His antibody; and by competition with 50-fold unlabeled DREs (Figure II-6*B*). Both rat and human 5-HT1A DREs effectively competed for the human Freud-1_L/probe complex in contrast to non-specific competitor (double-stranded E2F primers). Human Freud-1_L bound with greater affinity for human 5-HT1A DREs compared to the rat DREs (Figure II-6). Furthermore, two conjoined rat 5-HT1A DREs completely displaced the human Freud-1_L/probe complex, demonstrating higher affinity of Freud-1_L for the conjoined elements (Figure II-6*B*; lane 4). Thus, human Freud-1_L interacts with rat and human 5-HT1A DREs.

The interaction between endogenous Freud-1_L and the DREs of the 5-HT1A receptor gene was examined in human 5-HT1A-negative cells (SK-N-AS or A7) by chromatin immunoprecipitation assays (CHIP; Figure II-7). Freud-1/DNA complexes were immunoprecipitated using two different antibodies, anti-hFreud-1_L and anti-CC2D1A, which is specific for the amino-terminal of human Freud-1_L, which does not recognize Freud-1_S (Basel-Vanagaite et al., 2006). Elution fractions from both Freud-1 antibodies were specifically enriched in the 5-HT1A promoter region containing

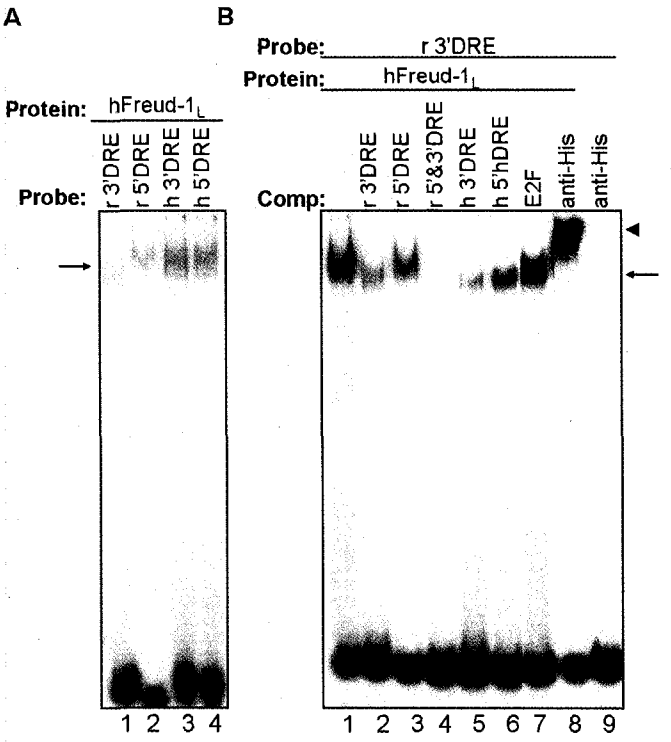

Figure II-6. Specific DNA-binding properties of human Freud-1_L.

(**A**) DRE-binding ability of human Freud-1_L. Recombinant, purified S/His-tagged human Freud-1_L (**hFreud-1_L**; 2 μg/sample) bound to radioactively-labeled double-stranded human (**h**) and rat (**r**) 5-HT1A DREs (Table II-I; 50,000 cpm/sample) as detected by EMSA (**arrow**). (**B**) Specificity of the human Freud-1_L/DRE complex. Purified S/His-tagged human Freud-1_L (**hFreud-1_L**; 2 μg/sample) was incubated with radioactively-labeled rat 5-HT1A 3'DRE (50,000 cpm/sample) in the absence or presence of 50-fold excess (ng/ng) of unlabeled double-stranded rat (**r**; lanes 2-4) or human (**h**; lanes 5 and 6) 5-HT1A DREs (**Comp**; Table II-I). The specificity of the complex (**arrow**) was also verified by supershift with anti-His antibody (1 μl; **arrowhead**).

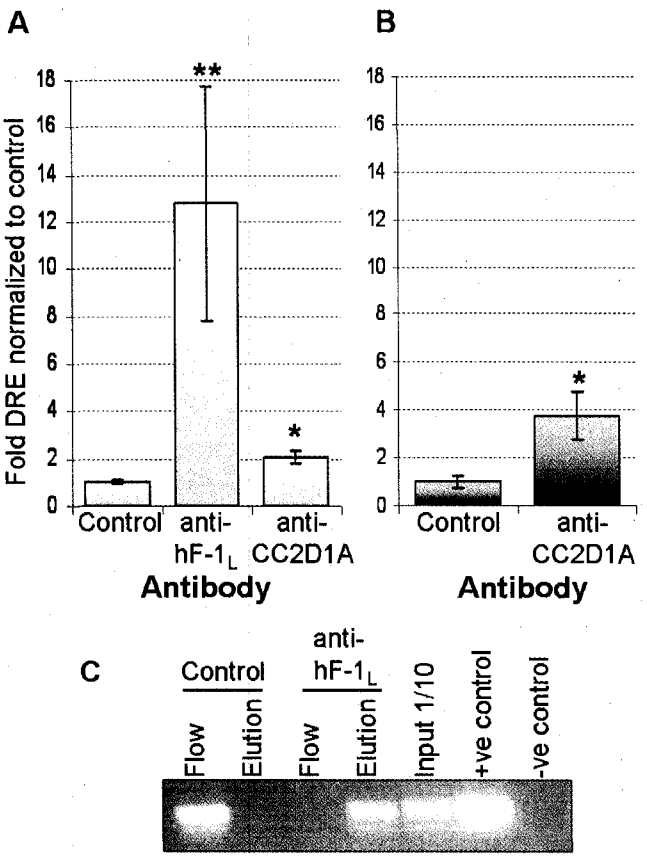

Figure II-7. Endogenous human Freud-1 is bound to the dual repressor elements of the 5-HT1A receptor gene.

Chromatin immunoprecipitation (CHIP) assays were performed on SK-N-AS (**A**) and A7 (**B**) cells. Cross-linked genomic DNA/Freud-1 complex was immunoprecipitated using anti-hFreud-1_L (**anti-hF-1_L**) and anti-CC2D1A antibodies. The elution fractions were analyzed by quantitative PCR with primers designed to amplify 151–bp region in the genomic DNA containing 5-HT1A 5'&3'DRE. Data are presented as -fold difference in C_T values adjusted to no antibody control (**control**) and shown as average ±S.E. (N≥3; **P=0.002 and *P=0.024, two-tailed unpaired t-test). (**C**) Semi-quantitative analysis of the CHIP assay performed on A7 cells. CHIP assays were done in the presence of anti-hFreud-1_L antibody (**anti-hF-1_L**) or preimmune serum (**control**). Elution, flowthrough (**Flow**), 1/10 input, 5-HT1A promoter plasmid (**+ve control**; PCR control) and no DNA negative control (**–ve control**; PCR control) were analyzed by PCR for genomic DNA region containing 5-HT1A 5'&3'DRE and products visualized following gel electrophoresis. The anti-hFreud-1_L antibody, but not preimmune, elution fraction was enriched in 5'&3'DRE (151-bp), indicating an endogenous Freud-1/DRE complex.

5'&3'DRE, as detected by quantitative PCR (QPCR) (Figure II-7*A*, *B*). A significant increase in PCR product compared to no antibody control (12.8-fold, P=0.002 and 2.1-fold, P=0.024 respectively) was observed in SK-N-AS immunoprecipitates using anti-hFreud-1_L and anti-CC2D1A antibodies (Figure II-7*A*). Similar results were observed in A7 cell using anti-CC2D1A antibody (3.7-fold enrichment, P=0.024; Figure II-7*B*). The specificity of the PCR product was demonstrated by semi-quantitative gel electrophoresis of products immunoprecipitated from A7 cells using anti-hFreud-1_L antibody. A single PCR product was detected in the anti-hFreud-1_L elution, but not in the preimmune antibody control (Figure II-7*C*). Furthermore, no amplification was present in the absence of DNA, while the specific PCR product was obtained using the 5-HT1A promoter plasmid or 1/10 input fraction (Figure II-7*C*). These results indicate that the endogenous human Freud-1_L is bound to the human 5-HT1A 5'&3'DRE in chromatin, consistent with a role for Freud-1_L in regulating the expression of the human 5-HT1A receptor gene.

2.5 DISCUSSION

Functional activity of human Freud-1_L at the dual repressor elements

In the current study we examined the regulation and function of a human Freud-1_L isoform and present several lines of evidence that support the role of human Freud-1_L as a repressor of the human 5-HT1A receptor gene. In previous studies, deletion of the DRE sites resulted in de-repression of the rat or human 5-HT1A promoters (Ou et al., 2000; Lemonde et al., 2004). Similarly, experiments depleting endogenous Freud-1 with an antisense construct upregulated 5-HT1A receptor expression (Ou et al., 2003), although relative contribution of the long or short Freud-1 isoform could not be conclusively determined. We show that human Freud-1_L is expressed in 5-HT1A-negative and -positive cells and shuttled out of the nucleus by CRM1/exportin 1-dependent nuclear export (Figure II-3). Freud-1_L is the predominant nuclear isoform in these cells (Figure II-3*B*) and binds to the human 5-HT1A DREs to mediate repression both *in vitro* (Figure II-5 and Figure II-6) and endogenously (Figure II-7) to repress 5-HT1A receptor gene transcription. In particular, our CHIP assays demonstrate the first evidence that Freud-1_L is bound to the DRE sites at the endogenous promoter of the 5-HT1A receptor gene (Figure II-7). Previous work has shown that DREs consist of 5'- and 3'-repressor elements, and the former is essential for Freud-1_S binding (Ou et al., 2003). The current results demonstrate that the tandem arrangement of two DREs that occurs in the 5-HT1A promoters is optimal for Freud-1_L binding (Figure II-6*B*), indicating that the sequence surrounding the DREs contributes to optimal binding. Our identification of the Freud-1_L-DRE interaction in cells that lack 5-HT1A receptor expression (Figure II-7), suggests that Freud-1_L participates in silencing 5-HT1A receptor gene. In non-neuronal cells, at least

three adjacent sites participate in silencing the 5-HT1A receptor gene: two DREs and a repressor element 1 (RE-1), which is recognized by a repressor element 1 silencing transcription factor (REST) (Lemonde et al., 2004). Hence, the interaction between Freud-1_L and the DRE in non-neuronal cells would contribute to silencing 5-HT1A receptor gene, but the knock-down of Freud-1 expression might not activate 5-HT1A receptor gene expression in non-neuronal cells contrary to cells that express endogenous 5-HT1A receptors (Ou et al., 2003). Taken together, these results indicate that human Freud-1_L is the major isoform that mediates repression of the human 5-HT1A receptor gene at the dual repressor elements.

Our data revealed that human Freud-1_L protein is present as 120/130-kDa doublet in the human cell lines analyzed (Figure II-3*B*). It is possible that the differences in size could be explained by specific post-translational modification such as SUMOylation (small ubiquitin-like modifier) or hyperphosphorylation, given that Freud-1 is predicted to have four putative SUMOylation sites as detected with SUMOplot™ (http://www.abgent.com/doc/sumoplot) and several putative phosphorylation sites (Ou et al., 2003). Further studies are necessary to define the basis for the observed molecular weight differences and their potential roles in Freud-1_L nuclear localization and repressor function. In this study we also detected a human Freud-1_S isoform that migrates at 67-kDa, while the predicted size is 43-kDa based on GenBank accession no. AK023399 (Figure II-1 and Figure II-3*A*, *B*). The discrepancy could result from an incorrect sequence in the database containing a premature stop codon.

Although Freud-1_L lacks a consensus nuclear localization sequence, it is predicted to be nuclear using LOCtree (http://cubic.bioc.columbia.edu/cgi-

bin/var/nair/loctree/query), an experimentally validated program that predicts cellular localization based on conserved structural features (Nair and Rost, 2005). In this study we observed different pattern of subcellular localization of Freud-1_L in three different cell lines including NG108-15, which showed low nuclear Freud-1_L immunoreactivity (Figure II-3*B*). This observation is consistent with previous report of primarily cytosolic distribution of human Freud-1_L in osteosarcoma cells (U2OS) as detected by immunocytochemistry (Basel-Vanagaite et al., 2006). We also show that the nuclear export of human Freud-1_L is dependent on CRM1/exportin 1 protein (Figure II-3*C*, *D*, *E*). Such nuclear export is observed for multiple transcription factors amongst which is a signal transducer and activator or transcription 2 (STAT2; (Banninger and Reich, 2004)) and v-rel reticuloendotheliosis viral oncogene homolog A (RelA; (Harhaj and Sun, 1999)). The role of cytosolic Freud-1 is unclear, but its export from the nucleus may play a role in calcium-mediated inactivation of Freud-1 (Ou et al., 2003).

Freud-1 and Mental Retardation

The broad tissue distribution of Freud-1 RNA is consistent with its role as a global repressor of many genes. Our Northern blot analysis of Freud-1 revealed similar tissue expression in humans (Figure II-2) and rodents (Ou et al., 2003; Basel-Vanagaite et al., 2006). In this study we detected three Freud-1 RNA species including a brain specific transcript (5-kb; Figure II-2). It is possible that the difference in Freud-1 RNA sizes could be attributed to variation in the polyadenylation sites.

Embryonic expression of Freud-1 in the cortical plate (day 12) precedes the expression of 5-HT1A receptor gene (Hillion et al., 1993; Basel-Vanagaite et al., 2006). The essential function of Freud-1 in the brain development is also supported by wide

distribution of Freud-1 in adult human brain (Figure II-2). Importantly, a deletion mutation truncating C-terminal half of the Freud-1 protein was recently linked to non-syndromic mental retardation (Basel-Vanagaite et al., 2006). The exact mechanism responsible for the mental retardation in Freud-1-linked families is currently unclear. However, it is possible to speculate that the mutant Freud-1 lacks DNA binding and repressor activity, given that the C2 domain was shown to be essential for these functions (Ou et al., 2003). Based on the current data the mutation could lead to an up-regulation in 5-HT1A receptor gene expression contributing to the altered neuronal development and cognitive impairment characteristic of mental retardation. For example, 5-HT1A receptors have been implicated in hippocampal neurogenesis (Santarelli et al., 2003; Banasr et al., 2004; Fricker et al., 2005) and their upregulation in hippocampus in mice during embryonic and perinatal development leads to reduced memory (Bert et al., 2005).

Freud-1 may repress other genes that have DNA elements homologous to the 5-HT1A DRE. For example, Freud-1 has been shown to repress dopamine-D2 receptor gene expression via a novel DRE (Rogaeva *et al.*, submitted), and thus altered regulation of the dopamine-D2 receptor may also contribute to the non-syndromic mental retardation phenotype of the Freud-1 mutation. Similarly, other Freud-1 gene targets could contribute to regulation of cognitive development by Freud-1. Sequence variations in genes encoding transcription factors or cofactors such as aristaless related homeobox (Gecz et al., 2006), SRY [sex determining region Y]-box 3 (Bergen et al., 2005), acrocephalosyndactyly 3 (TWIST; Saethre-Chotzen syndrome) (Gripp et al., 2000), CREB-binding protein (Partanen et al., 1999) and methyl CpG binding protein 2 (Johnston et al., 2001) have been associated with mental retardation. This suggests that

cognitive development is in part dependent on global regulation of gene expression. Altered expression of Freud-1 transcriptional targets in individuals homozygous for the Freud-1 mutation may be causative in the development of non-syndromic mental retardation, suggesting that Freud-1 is a critical regulator of normal mental health.

CHAPTER III - DIFFERENTIAL REPRESSION BY FREUD-1/CC2D1A AT A POLYMORPHIC SITE IN THE DOPAMINE-D2 RECEPTOR GENE

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Running title: Freud-1/CC2D1A regulation of the dopamine-D2 receptor gene

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FOOTNOTES

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ABBREVIATIONS

[1]The abbreviations used are: DRD2, dopamine-D2 receptor; 5-HT1A, serotonin 1A; DRE, Dual Repressor Element; D2-DRE, D2-Dual Repressor Element; FRE, 5'-Repressor Element; TRE, 3'-Repressor Element; 5-HT, serotonin; Freud-1, Five Prime Repressor Under Dual Repression Binding Protein 1; CC2D1A, coiled-coil and C2 domain containing 1A; CHIP, Chromatin Immunoprecipitation; EMSA, Electrophoretic Mobility Gel Shift Assay; siRNA, small interfering RNA; QPCR, quantitative PCR; DM14, *Drosophila melanogaster* 14; C2, protein kinase C conserved region 2; mFreud-1, mouse Freud-1; DMEM, Dulbecco's Modified Eagle's Medium; MEM, Minimum Essential Medium; IPTG, isopropyl-β-D-thiogalactopyranoside; anti-hFreud-1, anti-human Freud-1Long antibody; GAPDH, glyseraldehyde-3-phosphate dehydrogenase; PBS, Phosphate Buffered Saline; ADHD, attention deficit hyperactivity disorder; ATRX, alpha thalassemia/mental retardation syndrome X-linked; MeCP2, methyl CpG binding protein 2; DEAF-1, deformed epidermal autoregulatory factor 1; FCS, Fetal Calf Serum.

Authors' contributions: I have performed most of the work depicted in this manuscript including the writing of the preliminary manuscript. Dr. Paul Albert and I have edited the follow up versions, prepared it for submission as well as addressed reviewer's comments. Dr. Xiao-Ming Ou did an experiment demonstrating the effect of the polymorphism (rs2734836) on Freud-1 binding (Figure III-4*D*). Dr. Hamed Jafar-Nejad has done the preliminary analysis of the repressor element; however, none of his immediate work is presented in this manuscript. Dr. Sylvie Lemonde has contributed to this manuscript through teaching me techniques and helping me troubleshoot at the initial stages of the project.

3.1 ABSTRACT

Freud-1/CC2D1A is a transcriptional repressor of the serotonin-1A receptor gene and was recently genetically linked to non-syndromic mental retardation. To identify new Freud-1 gene targets, data base mining for Freud-1 recognition sequences was done. A highly homologous intronic element (D2-DRE) was identified in the human dopamine-D2 receptor (*DRD2*) gene, and the role of Freud-1 in regulating the gene at this site was assessed. Recombinant Freud-1 bound specifically to the D2-DRE, and a major protein-D2-DRE complex was identified in nuclear extracts that was supershifted using Freud-1-specific antibodies. Endogenous Freud-1 binding to the D2-DRE in cells was detected using chromatin immunoprecipitation. The D2-DRE conferred strong repressor activity in transcriptional reporter assays that was dependent on the Freud-1 recognition sequence. In three different human cell lines, the level of Freud-1 protein was inversely related to DRD2 expression. Knockdown of endogenous Freud-1 using small interfering RNA resulted in an up-regulation of DRD2 RNA and binding sites, demonstrating a crucial role for Freud-1 in DRD2 regulation. A previously uncharacterized single nucleotide A/G polymorphism (rs2734836) was located adjacent to the D2-DRE and conferred allele-specific Freud-1 binding and repression, with the major G-allele having reduced activity. These studies demonstrate a key role for Freud-1 to regulate DRD2 expression and provide the first mechanistic insights into its transcriptional regulation. Allele-specific regulation of DRD2 expression by Freud-1 may possibly associate with psychiatric disorders or mental retardation.

3.2 INTRODUCTION

Dopamine-D2 receptors function as both pre-synaptic autoreceptors and post-synaptic receptors, and play key roles in regulating dopaminergic neurotransmission. Increased levels of dopamine-D2 receptors or dopaminergic hyperactivity have been implicated in schizophrenia (Abi-Dargham et al., 2000; Seeman and Kapur, 2000; Gainetdinov et al., 2001), and most antipsychotic drugs inhibit dopamine-D2 receptors (Missale et al., 1998; Lewis and Levitt, 2002; Miyamoto et al., 2005). Although transcriptional regulation of the rat dopamine D2 receptor (*DRD2*)[1] gene has been examined, very little is known regarding the regulation of the human *DRD2* gene. A polymorphism in the putative *DRD2* promoter confers decreased transcriptional activity and has been negatively associated with schizophrenia (Arinami et al., 1997). However, the transcriptional mechanisms for regulation of the human *DRD2* gene have yet to be elucidated.

In order to identify new transcriptional regulators in the nervous system, we previously characterized the serotonin-1A (5-HT1A) receptor promoter region and identified a novel dual repressor element (DRE) that negatively regulates its expression (Ou et al., 2000; Lemonde et al., 2004). The DRE consists of adjacent and partially overlapping repressor elements: 5'-repressor element (FRE; major regulator in neuronal cells) and 3'-repressor element (TRE) (Ou et al., 2000). Analysis of DRE-binding proteins revealed that a novel protein, Freud-1 (Five prime Repressor Under Dual repression binding protein-1)/CC2D1A (Coiled-coil and C2 Domain containing 1A) binds to and represses the 5-HT1A receptor gene through the FRE (Ou et al., 2003). In 5-HT1A-expressing raphe cells, inactivation of Freud-1 by calcium-calmodulin kinase or using antisense to Freud-1 leads to up-regulation of 5-HT1A receptor expression. Thus,

Freud-1 helps to establish the basal level of 5-HT1A receptor expression in raphe neurons.

Freud-1 is evolutionary conserved and contains a variable number of *Drosophila melanogaster* 14 repeats (DM14), a helix-loop-helix and a C2 (protein kinase C conserved region 2) calcium-phospholipid binding domain (Ou et al., 2003; Albert and Lemonde, 2004). Recently, linkage analysis in patients with autosomal recessive non-syndromic mental retardation has revealed a deletion mutation that eliminates exons 14 to 16 of the *CC2D1A/Freud-1* gene and encodes a truncated protein lacking the fourth DM14, helix-loop-helix and C2 domains (Basel-Vanagaite et al., 2006). The C2 domain is essential for Freud-1 DNA binding and repressor functions, implying that the mutated Freud-1 protein is non-functional (Ou et al., 2003). Linkage of the *CC2D1A/Freud-1* gene with non-syndromic mental retardation and its widespread localization in brain, including dopaminergic neurons (Ou et al., 2003; Basel-Vanagaite et al., 2006) suggested that Freud-1 may regulate other genes in addition to 5-HT1A receptors (Rogaeva et al., 2007a).

In this study, database mining identified a highly conserved DRE sequence in the second intron of the *DRD2* gene (D2-DRE) and two proximal and previously uncharacterized polymorphisms. We then investigated allele-dependent binding and repression of the D2-DRE by Freud-1 and the role of Freud-1 in regulation of dopamine-D2 receptor expression. We find that Freud-1 functions as a transcriptional regulator of the human *DRD2* gene.

3.3 MATERIALS AND METHODS

Plasmid constructs - Cloning of mouse Freud-1 (mFreud-1; NCBI accession #ABC56419) into pET30A and pcDNA3 (Invitrogen, Burlington, ON) has been previously described (Ou et al., 2003). Luciferase reporter constructs containing D2-DRE incorporated primers containing the A-allele (D2-DRE(A), G-allele D2-DRE(G) for rs2734836 (5'-cgcgtgggataagcaagcccttcttgtaaaagtttaagaac(**g/a**)ataca-3') or the mutant D2-DRE(m3) (5'-cgcgtgg**acat** ag**agca**ccctt**tgta**taaaagttttaagaacaataca-3') flanked by MluI sites were generated. The complementary D2-DRE primers were purified by electrophoresis on 19% polyacrylamide gel, annealed, and subcloned into MluI-digested pGL3-Promoter vector (Promega, Madison, WI) and verified by DNA sequencing.

Cell culture, Transient Transfections, and Luciferase Reporter Assays – Human cell lines were maintained at 37°C in 5% CO_2. HEK293 (embryonic kidney) and SK-N-AS cells (bone marrow neuroblastoma (Wang and Bannon, 2005); a gift from Dr. Michael Bannon, Wayne State Univ., Detroit, MI) were grown in Dulbecco's Modified Eagle's Medium (DMEM; Wisent, St-Bruno, QC) supplemented with 10% Fetal Calf Serum (FCS). Y-79 cells (retinoblastoma; ATCC, Manassas, VA) were grown in RPMI 1640 supplemented with 20% FCS, 10 mM HEPES, 1 mM sodium pyruvate and 4.5 g/L glucose (Wisent). A7 (melanoma cells stably transfected with filamin 1 (Lin et al., 2001)), obtained from Dr. John Hartwig (Brigham and Women's Hospital, Boston, MA), were grown in Minimum Essential Medium (MEM; Wisent) supplemented with 8% Newborn Calf Serum, 2% FCS, and 500 µg/ml of G418 (Invitrogen).

All cell lines were grown to 50-60% confluence, and the media replaced 12 h before transfection. HEK293 and A7 cells were transfected with 20 µg of reporter

construct and 5 μg of pCMV-βgal (Clontech, Mountain View, CA) using calcium phosphate coprecipitation as described previously (Storring et al., 1999; Ou et al., 2000). All plasmids were purified by maxiprep kit (Sigma, St. Louis, MO), quantified spectrophotometrically and verified by ethidium bromide staining. For reporter assays, triplicate samples were extracted with 200 μl of Reporter Lysis Buffer (Promega) and supernatants were collected and assayed for luciferase activity using Promega Luciferase Assay system by 1250 luminometer (Bio Orbit, Turku, Finland) and normalized to β-galactosidase activity using 4-methyl-umbelliferyl-β-D galactoside (Sigma) conversion to methylumbelliferone (λex=350 nm, λem=450 nm), and activity normalized to that of pGL3-Basic vector (Promega).

Production and Purification of Recombinant Freud-1 - BL21 (DE3) E. coli was transformed with Freud-1 expression plasmid pET30A-mFreud-1, grown overnight and induced at OD_{600}=0.6 with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Wisent) at 37°C for 3 h. Cells were harvested and mFreud-1 purified by TALON metal affinity resin (Promega). *In vitro* transcription/translation of mFreud-1 was performed using TNT® Coupled Reticulocyte Lysate System (Promega) with 1 μg of pcDNA3-mFreud-1.

Electrophoretic Mobility Gel Shift Assay (EMSA) - Complementary D2-DRE oligonucleotides used for reporter constructs were annealed and end-labeled with [α-^{32}P] dCTP using 2.5U Klenow (New England Biolabs, Pickering, ON) and purified over Sephadex G-50 column (GE HealthCare, Baie d'Urfe, QC). Nuclear extracts (5 μg/sample), *in vitro* transcribed/translated mFreud-1 (5 μl/sample), or purified mFreud-1 (4 μg/sample) were preincubated with or without double-stranded competitor DNA: D2-DRE(A), D2-DRE(G), D2-DRE(m3), E2F (5'-tatagtgtactcta ctattctgctc-3'), 5-HT DRE

(5'-cggcataagcaagccctt attgcacagagc-3'), antibodies (1 µl of anti-mFreud-1, rabbit IgG and anti-CC2D1A with or without 1x blocking peptide (Bethyl Laboratories Inc., Montgomery, TX)) were used in a 25 µl reaction containing DNA binding buffer (20 mM HEPES, 0.2 mM EDTA, 0.2 mM EGTA, 100 mM KCl, 5% glycerol and 2 mM DTT, pH 7.9) and 2 µg of poly (d[I-C]) or 250 ng of herring sperm DNA (Roche, Laval, QC) and incubated at room temperature for 30 min. ^{32}P-labeled probe (50,000 cpm/sample) was then added and incubated for an additional 20 min at room temperature. The DNA/protein complexes were separated on a 5% polyacrylamide gel at 4°C dried and exposed to film (Ou et al., 2000).

Antibodies, Western Blot, and Chromatin Immunoprecipitation (CHIP) - Previously described rabbit anti-mFreud-1 antibody was used for supershift in the EMSA (Ou et al., 2003). Polyclonal rabbit anti-human Freud-1Long antibody (anti-hFreud-1; 1:20,000) was raised (Cedarlane, Hornby, ON) against bacterially expressed and purified (Ni-NTA beads; Qiagen, Mississauga, ON) S-/His-tagged hFreud-1Long (pTriEX4 vector; Novagen, Madison, WI) as antigen (NCBI Accession #Q6P1N0). Anti-CC2D1A antibody was used in CHIP assays (1:1,000 dilution) and in supershift EMSA, in the presence or absence of its specific blocking peptide (Bethyl Laboratories), which was preincubated with the antibody overnight at 4°C. In addition, anti-β-actin (1:20,000; Sigma), anti-cRaf (1:3,000; BD Biosciences, Mississauga, ON) and anti-Histone H1 (1:1,000; Upstate Biotechnology, Lake Placid, NY) were used as controls. Immunoblots were performed as described previously (Ghahremani et al., 1999). Membranes were incubated with primary antibodies overnight at 4°C, followed by horseradish peroxidase-linked anti-rabbit (1:2,000; New England Biolabs) or anti-mouse secondary antibody

(1:2,000; Jackson Immunoresearch Laboratories, West Grove, PA) and tertiary BM chemiluminescence blotting substrate (Roche). Specificity of anti-hFreud-1 antibody was assessed by Western blot and immunoprecipitation analyses, where a ~130-kDa band was identified only with immunized serum (Figure III-1) and verified by mass spectrometry (Ottawa Genomics Innovation Center Proteomics Facility; data not shown).

CHIP assays were performed as described in Upstate protocol (Upstate) with modifications. Cells were washed 3x with Phosphate Buffered Saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) and crosslinked for 15 min at room temperature in PBS supplemented with 1 mM $MgCl_2$ and 1% formaldehyde (v/v), rinsed 3x with PBS and lysed (Nowak et al., 2005). Shearing of genomic DNA (100- to 400-bp fragments; data not shown) was done by sonication on ice with addition of 212- to 300-μm diameter glass beads (Sigma) (Weinmann et al., 2001), 15x at the setting of 7 for 20 s each time (60 Sonic Dismembrator, Fisher, Ottawa, ON). De-crosslinking was done overnight at 65°C, followed by 1 h digestion with proteinase K (Sigma) and phenol/chloroform extraction. The results were analyzed using quantitative PCR (QPCR; Rotor-Gene RG-3,000; Corbett Life Science, Sydney, Australia) with two sets of oligonucleotide primers: one designed to amplify a 124-bp (5'-ctac tgtgggcattgcactttat-3' and 5'-tgaacttggccacgttacatg -3') and the other a 206-bp region (5'-ccttccagggc agcagcttagtag-3' and 5'-ataagacatctactgtgggcattg-3') containing D2-DRE. The PCR reaction was performed using qTaq™ DNA polymerase mix (Clontech) and amplification cycles were: 92°C for 10 min; 92°C for 30 s; 54°C for 30 s; 72°C for 30 s; 84°C for 20 s (25 cycles), and terminated at 72°C for 10 min. The results were visualized

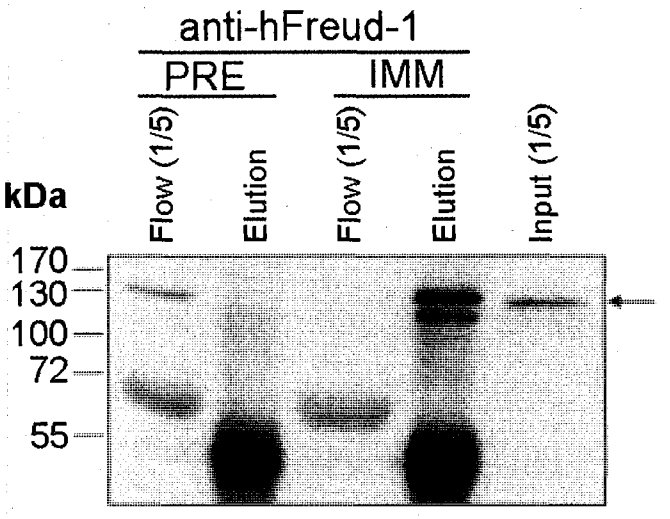

Figure III-1. Specificity of anti-hFreud-1 antibody.

Anti-hFreud-1 antibody immunoprecipitates Freud-1 from A7 cells (~130-kDa, the molecular weight of hFreud-1; **arrow**) as demonstrated by comparing preimmune (**PRE**) and immunized (**IMM**) serum. A fraction of each immunoprecipitation step (input, flowthrough (**Flow**) and elution) was examined on the Western blot (**bracket**) using anti-hFreud-1 antibody (1:20,000) for Freud-1 detection. The identity of the band was further validated by mass spectrometry (data not shown).

using SYBR green (Molecular Probes, Eugene, OR) incorporation and verified on agarose gel.

Immunoprecipitation - Cells were rinsed 2x with ice cold PBS and lysed in modified RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 2 mM EDTA) supplemented with 1x protease inhibitor cocktail (Roche) on ice for 30 min followed by sonication (60 Sonic Dismembrator, Fisher) once at the setting of 3 for 10 s. Lysates were centrifuged at 10,000Xg for 30 min at 4°C and the supernatant was diluted 10x with PBS/protease inhibitor cocktail. The lysates were precleared with 40 µl of protein A agarose beads (GE HealthCare) with rotation at 4°C for 30 min and combined with anti-hFreud-1 antibody (1:1,000) overnight at 4°C. The following day 20 µl of protein A agarose beads were added and incubated (2 h, 4°C). The supernatant was then collected and the beads washed with 1 ml of NETIN buffer (20 mM Tris pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.5% NP-40) 3x with gentle inversion and spun for 10 s at 1,000Xg. The beads were then boiled for 5 min in 2x Loading buffer (200 mM Tris pH 6.8, 0.8% SDS, 1.6% 2-mercaptoethanol, 0.04% Bromophenol blue, 4% glycerol) and subjected to Western blot analysis.

Nuclear and Cytosolic Fractionation and siRNA - Nuclear/cytosolic fractionation was performed as previously described (Czesak et al., 2006). Briefly, cells were lysed on ice with extraction buffer (10 mM KCl, 10 mM Na-HEPES, pH 7.6, 1.5 mM $MgCl_2$, 0.1% NP-40, 0.5 mM DTT, 0.5 mM spermidine, 0.15 mM spermine, 1 mM PMSF, 1x Protease Inhibitor Cocktail (Roche)) for 10 min. The resultant nuclei were washed (50 mM NaCl, 20 mM Na-HEPES, pH 7.6, 25% glycerin, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM spermidine, 0.15 mM spermine, 1 mM PMSF, 1x Protease Inhibitor Cocktail)

and lysed with nuclear extraction buffer (500 mM NaCl, 20 mM Na-HEPES, pH 7.6, 25% glycerin, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM spermidine, 0.15 mM spermine, 1 mM PMSF, 1x Protease Inhibitor Cocktail) and analyzed for success of fractionation and presence of Freud-1 by Western blot analysis.

Stealth siRNA targeting hFreud-1 (5'-ggc gcucuaucagacagcaauugaa-3') and a scrambled negative control (5'-ggcucucuaagagacaacuugcgaa-3') were designed online (https://rnaidesigner. invitrogen.com/sirna/; Invitrogen). A7 and SK-N-AS cells were transfected using HiPerFect (Qiagen) transfection reagent and Y-79 cells using Lipofectamine™ 2000 (Invitrogen) for Y-79, with a final siRNA concentration of 20 nM and 33 nM, respectively. Transfection efficiency control was performed with BLOCK-iT™ Fluorescent Oligo (Invitrogen) demonstrating ~90% efficiency (data not shown). The cells were analyzed 72 h post transfection.

Dopamine-D2 ligand binding assay - Dopamine-D2 receptor sites were measured by specific binding of the antagonist [^{3}H]spiperone (119 Ci/mmol; Amersham, Baie d'Urfe, QC, CA). Membranes were prepared from A7, SK-N-AS and Y-79 cells as previously described (Kushwaha et al., 2006). Membranes were diluted with 1/5 TME buffer (15 mM Tris-HCl, pH 7.4, 2.5 mM $MgCl_2$, and 0.2 mM EDTA) supplemented with 0.1% ascorbic acid and 9,000 cpm of [^{3}H]spiperone in the presence or absence of apomorphine (10^{-6} M; Sigma). After 30 min incubation at room temperature the samples were filtered through GF/C glass microfiber filters (Whatman, Clifton, NJ, USA) and washed 3x with 5 ml of ice-cold 50 mM Tris pH 7.4. The filters were then combined with 3 ml of scintillation fluid (InterSciences Inc., Markham, ON, CA) and the radioactivity detected using Packard TRI-CARB 2100TR scintillation counter (PerkinElmer, Woodbridge, ON,

CA). The receptor binding was normalized to protein concentration determined by bicinchoninic acid assay (Pierce, Nepean, ON, CA).

Preparation of RNA, cDNA and QPCR analysis - The RNA was isolated using TRIZOL® Reagent (Invitrogen) followed by DNAse treatment with TURBO DNA-*free*™ kit (Ambion, Austin, TX) and cDNA was generated using the Cells-to-cDNA™ II kit (Ambion). The resulting cDNA was analyzed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; control) and dopamine-D2 expression levels by QPCR analysis and SYBR green detection method. More specifically, amplification cycles were: 95°C for 3 min, 92°C, 20 s; 60°C, 20 s; 72°C for 20 s (40 cycles); and terminated at 72°C for 15 min performed with PCR primers (IDT, Coralville, IA) for human GAPDH (5'-cgacagtcagccgcatcttctttt-3' and 5'-gcgc ccaatacgaccaaatcc-3') and human dopamine-D2 receptor cDNA (5'-tcgtcgccacactggtcatgc-3' and 5'-gcttggagctgtagcgcgtattgta-3'). The resulting PCR products were analyzed as previously described, (Livak and Schmittgen, 2001) using the $2^{-\Delta\Delta C_T}$ method, validated by analyzing ΔC_T ($C_{T,hFreud-1}$-$C_{T,GAPDH}$), which produced a slope of 0.0633 (slope<0.1 validates the use of this method). In addition, PCR products were verified on agarose gel (data not shown).

3.4 RESULTS

Identification and function of the D2-DRE

In order to identify new DNA elements recognized by Freud-1, a Blast Search was performed using the rat 5-HT1A 3'DRE sequence, a known Freud-1 binding site (Ou et al., 2000). A highly homologous sequence was recognized in the second intron of the *DRD2* (D2-DRE; position 12432-12461, GenBank #AF050737). The D2-DRE displayed strong nucleotide similarity (80.6%) to the rat 5-HT1A 3'DRE (Figure III-2*A*), with highest homology (95.7%) within the 5'-Repressor Element (FRE), required for Freud-1 protein binding (Ou et al., 2000). Furthermore, the level of interspecies similarity of mammalian D2-FRE sites was similar to that between the functional FREs of human and rat 5-HT1A receptor genes (Figure III-2*B*), suggesting a conserved role for this site. Because of its conserved sequence, the D2-DRE was examined further as a potential Freud-1 regulatory element of the *DRD2* gene.

To determine whether nuclear proteins bind to the D2-DRE, nuclear extracts from HEK293 or dopamine-D2 receptor-positive A7 cells and labeled complementary D2-DRE oligonucleotides were co-incubated and examined by electrophoretic mobility gel shift assays (EMSA; Figure III-3*A*). In both cell types, a major protein/D2-DRE complex was observed (arrow) that was competed with excess unlabeled D2-DRE double-stranded oligonucleotides, but not unrelated double-stranded E2F primers. Mutant D2-DRE(m3) double-stranded oligonucleotides incorporating mutations shown to reduce Freud-1 binding to the 5-HT1A 3'DRE (Ou et al., 2000), did not compete for the major protein/D2-DRE complex compared to the non-mutated D2-DRE. The DNA sequence

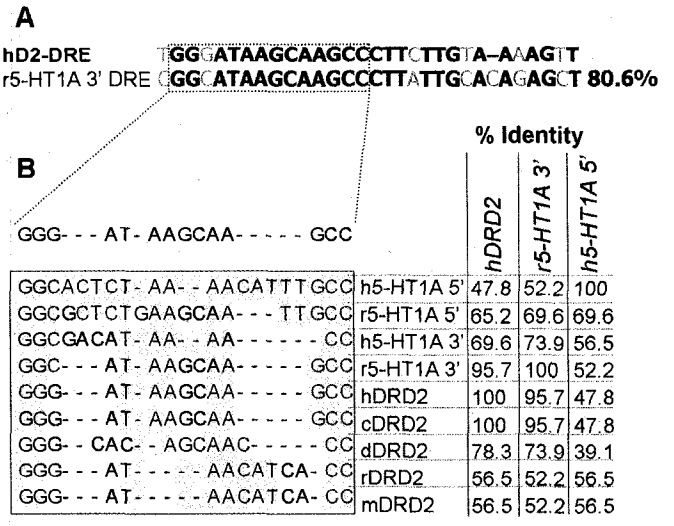

Figure III-2. Dual repressor elements of the 5-HT1A and dopamine-D2 receptor genes. GenBank accession numbers and position of the first nucleotide are shown in **[brackets]**. (**A**) Sequence alignment of D2-DRE in the second intron of the human *DRD2* [AF050737, 12432] with rat 5-HT1A 3'DRE [AF087675, 2058]. Conserved base pairs are in bold; Freud-1 binding site (5'-Repressor Element) is boxed. (**B**) Alignment of Freud-1 recognition DNA sequences. Conserved nucleotides, as compared to human 5-HT1A 5'FRE (**h5-HT1A 5'** [AC122707, 101949]), are highlighted in gray. Nucleic acid consensus is depicted at the top of the sequences. Similarity to human DRD2 FRE (**hDRD2** [AF050737, 12433]), rat 5-HT1A 3'FRE (**r5-HT1A 3'** [AF087675, 2059]) and h5-HT1A 5' is shown as percent identity. FRE sequences: human 5-HT1A 3'FRE (**h5-HT1A 3'** [AC122707, 101976]), rat 5-HT1A 5'FRE (**r5-HT1A 5'** [AF087675, 2029]), chimpanzee DRD2 FRE (**cDRD2** [NM 001033928]), dog DRD2 FRE (**dDRD2** [NM 001003110, 2860]), rat DRD2 FRE (**rDRD2** [NM 012547, 57031]) and mouse DRD2 FRE (**mDRD2** [NM010077, 58381]).

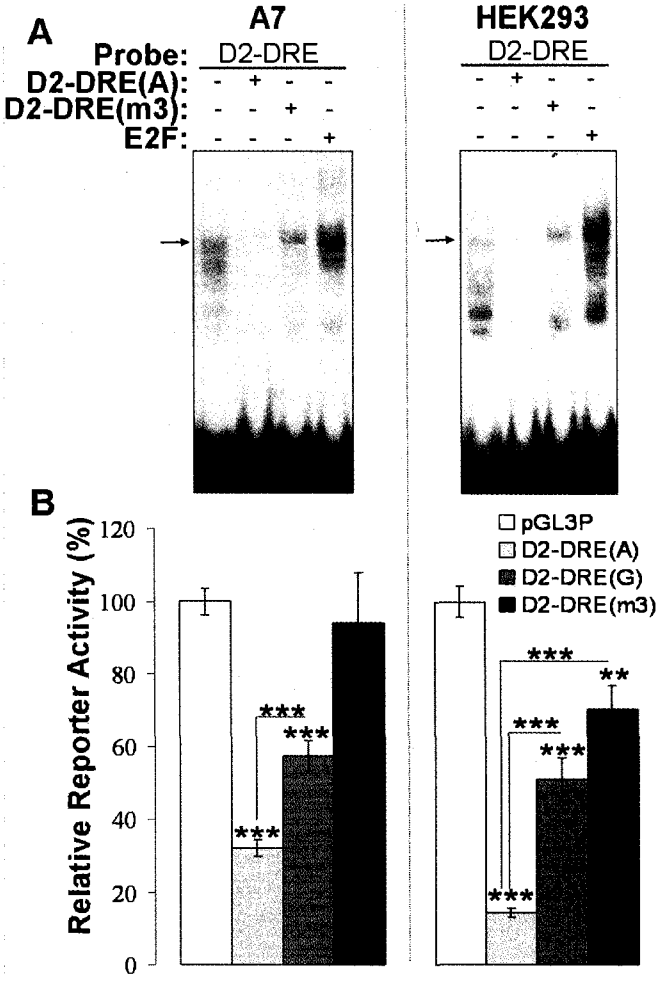

Figure III-3. Protein binding and allele-specific repressor activity of the D2-DRE.

(**A**) Binding of a nuclear protein complex to the D2-DRE. EMSA was performed using A7 or HEK293 nuclear extracts (5 µg/sample) and ^{32}P-labeled D2-DRE (50,000 cpm/sample), and competed with either 200x (for HEK293) or 50x (for A7 cells) molar excess of unlabeled double-stranded D2-DRE with either A-allele (D2-DRE(A)), mutationally inactivated D2-DRE (D2-DRE(m3)), or unrelated E2F primers. A major specific protein/DNA complex (arrow) was detected in the presence but not absence (data not shown) of nuclear extracts. (**B**) Allele-specific repressor activity of D2-DRE in A7 and HEK293 cells. Luciferase constructs used contained SV40 promoter alone (pGL3P;

empty vector) or D2-DRE cloned 5' to SV40 promoter as A-allele (D2-DRE(A)), G-allele (D2-DRE(G)) or mutant D2-DRE(m3) (Ou et al., 2000). Relative reporter activity was normalized to β-galactosidase activity and is depicted as percent of vector activity, average ±S.E. ($N \geq 4$). ***$P \leq 0.0005$, **$P \leq 0.005$ (two-tailed unpaired t-test).

specificity of the major protein/D2-DRE complex is consistent with the binding of Freud-1 to the D2-DRE.

The transcriptional regulatory activity of the D2-DRE was examined using luciferase reporter assays. A7 and HEK293 cells transiently transfected with D2-DRE-containing plasmid displayed reduced luciferase activity compared to vector-transfected cells, indicating that the D2-DRE confers repressor activity (Figure III-3*B*). Similarly, strong repressor activity was observed when D2-DRE was placed downstream of the SV40 promoter (data not shown), supporting its role as a position-independent repressor element. The D2-DRE(m3) mutant construct lacked significant activity in A7 cells and marginally reduced transcriptional activity in HEK293 cells, consistent with the weak protein binding activity of this mutant element (Figure III-3*A*). These results indicate that the sequence specificity of the D2-DRE conforms to a Freud-1 binding site. Taken together, the protein binding and repressor activities of the D2-DRE suggest its role as a repressor element of the *DRD2* gene.

Analysis of the sequence surrounding the D2-DRE (http://www.ncbi.nlm.nih.gov/SNP/) revealed two previously uncharacterized single nucleotide polymorphisms: A/G (rs2734836) and A/C (rs2734835). The more proximal polymorphism (rs2734836) is located 8-bp downstream of the D2-DRE. The effect of the rs2734836 polymorphism on D2-DRE repressor activity was analyzed using reporter constructs incorporating D2-DRE with either A- or G-allele (D2-DRE(A) or (G)). Although both D2-DRE alleles displayed significant repression in A7 cells, the G-allele exhibited significantly less repression than the A-allele. Similarly in HEK293 cells the A-allele repressed luciferase activity to below 20% of control, while the G-allele displayed

only 50% repression (Figure III-3*B*). These data indicate that the A-allele of the rs2734836 polymorphism has a stronger repressor activity in both cell lines than the G-allele.

Freud-1 interacts with the D2-DRE

In order to address whether Freud-1 is present in the protein/D2-DRE complex from HEK293 nuclear extracts, an antibody specific for Freud-1 (anti-mFreud-1 (Ou et al., 2003)) was included to supershift the complex (Figure III-4*A*). In the presence of anti-mFreud-1 antibody but not preimmune serum, a slowly migrating protein/D2-DRE complex was observed (solid arrowhead), consistent with the presence of antibody-bound Freud-1 in the complex. Similarly, incubation of nuclear extracts from A7 cells with anti-CC2D1A antibody (human Freud-1) resulted in a mobility shift of the protein/D2-DRE complex (Figure III-4*B*, solid arrowhead). The super-shifted complex was partially displaced by inclusion of antigenic blocking peptide, indicating the specificity of the antibody. These results indicate that Freud-1 is present in nuclear extracts and binds to the D2-DRE.

We examined whether Freud-1 directly interacts with the D2-DRE using *in vitro* transcribed and translated mouse Freud-1 protein. Incubation of recombinant Freud-1 with labeled D2-DRE revealed a specific protein/DNA complex that was efficiently competed by excess unlabeled D2-DRE (Figure III-4*C*, arrow), but not by unrelated E2F primers (data not shown), indicating that Freud-1 binds directly to the D2-DRE. The influence of the rs2734836 polymorphism on the binding of recombinant Freud-1 was determined by EMSA with either the labeled A- or G-allele of the D2-DRE (Figure III-4*D*). The intensity of the Freud-1/D2-DRE complex was greater for D2-DRE(A) than

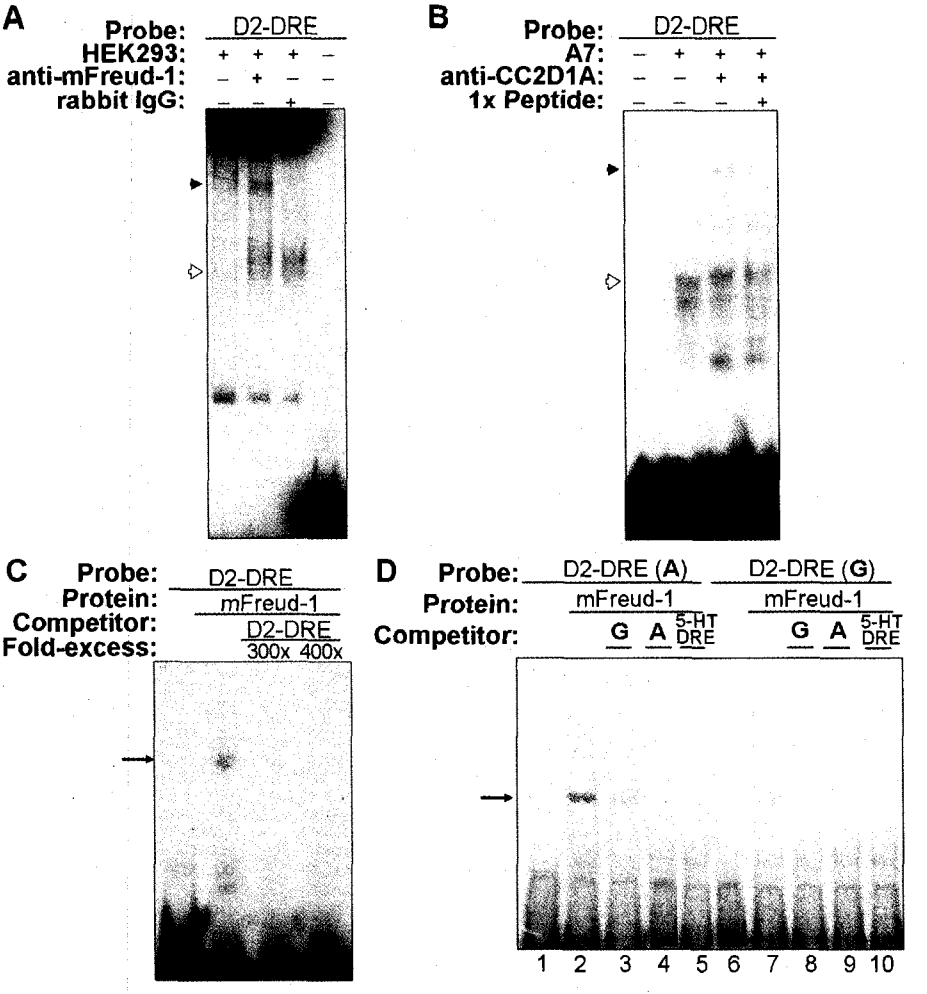

Figure III-4. Freud-1 binds to the D2-DRE.

(**A**) Nuclear extracts from HEK293 cells were analyzed by EMSA using radioactively labeled D2-DRE probe. A specific protein/D2-DRE complex (**open arrowhead**) was supershifted in the presence of 1 µl anti-mFreud-1 antibody (**solid arrowhead**), but not in the presence of rabbit IgG control. (**B**) The presence of Freud-1 in the protein/D2-DRE complex from A7 nuclear extracts. Supershift (**solid arrowhead**) of the protein/D2-DRE complex (**open arrowhead**) was observed in the presence of 1 µl of anti-CC2D1A antibody and reduced by addition of 1x (mass/mass) Freud-1 antigenic peptide control. (**C**) Recombinant Freud-1 specifically binds to the D2-DRE. Complementary end-labeled

D2-DRE primers (**D2-DRE**) were incubated with *in vitro* transcribed/translated mouse Freud-1 (**mFreud-1**). A single specific protein/DNA complex was detected (**arrow**) that was not observed in the absence of Freud-1 or in samples with 300- or 400-fold excess (**300x, 400x,** ng/ng) unlabeled D2-DRE. (**D**) Allele-specific binding of Freud-1 to the D2-DRE. Recombinant mouse Freud-1 was incubated with probes for the D2-DRE A-allele (**D2-DRE(A)**; lanes 1 to 5) or G-allele (**D2-DRE(G);** lanes 6 to 10) in EMSA, without or with 100-fold competitor (unlabeled D2-DRE(**A**), D2-DRE(**G**) or rat 5-HT1A 3'DRE (**5-HT DRE**)). **Arrow** indicates the Freud-1/D2-DRE complex.

for D2-DRE(G), suggesting a greater binding affinity of Freud-1 for the A-allele (Figure III-4*D*; lane 2 vs. 7). Consistent with this, a 100-fold excess of unlabeled A-allele completely displaced DRE binding, while the G-allele was only partially effective (Figure III-4*D*; lane 3 vs. 4). Unlabeled rat 5-HT1A 3'DRE oligonucleotides competed as effectively as the D2-DRE A-allele, consistent with specific binding of Freud-1 to both DRE sites (Figure III-4*D*). These results demonstrate that the G-allele of the rs2734836 polymorphism displays reduced affinity for Freud-1 binding, correlating with a decrease in Freud-1-mediated repression at the G-allele compared to the A-allele (Figure III-3*B*).

To address whether endogenous Freud-1 is bound to the *DRD2* gene in cells, quantitative chromatin immunoprecipitation (CHIP) assays were conducted. Anti-CC2D1A antibody was used to immunoprecipitate Freud-1/DNA complexes from cell lysates and D2-DRE content was measured by quantitative PCR (QPCR) analysis. A statistically significant enrichment of the D2-DRE from HEK293 cells in the elution fractions was found upon immunoprecipitation using anti-CC2D1A antibody compared to no antibody control (Figure III-5*A*). Similar results were obtained using anti-hFreud-1 antibody (data not shown). Similarly, in DRD2-expressing SK-N-AS cells, anti-CC2D1A antibody immunoprecipitated a D2-DRE-containing complex from chromatin as demonstrated by gel electrophoresis of the elution fractions (Figure III-5*B*). Preincubation with antigenic Freud-1 peptide reduced the immunoprecipitation of D2-DRE/Freud-1 complex (Figure III-5*B*; lane 3), confirming the specificity of the CHIP assay. These results demonstrate specific binding of endogenous Freud-1 protein to the D2-DRE of the second intron of the dopamine-D2 receptor gene.

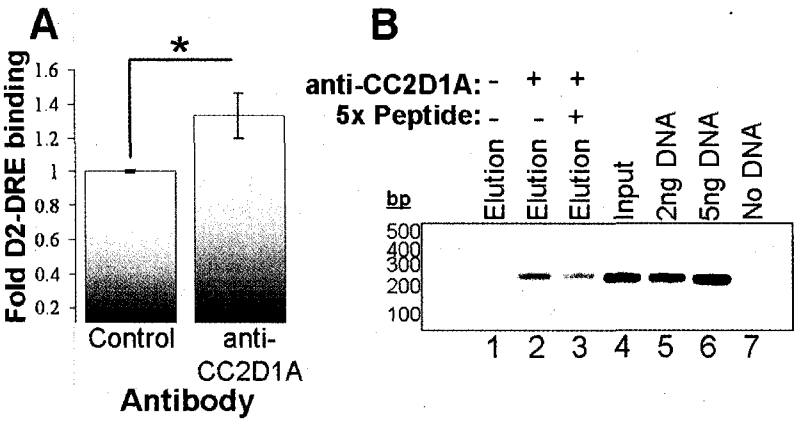

Figure III-5. Interaction of endogenous human Freud-1 with the D2-DRE in genomic DNA revealed through CHIP assays.

(**A**) Elution fractions from HEK293 cell lysates in the absence (**Control**) or presence of Freud-1 specific antibody (**anti-CC2D1A**) were quantified by qPCR analysis for D2-DRE containing genomic DNA. Data are presented as -fold difference in C_T values adjusted to control and shown as average ±S.E. (N=3). *$P \leq 0.05$ (two-tailed unpaired t-test). (**B**) Results of CHIP assay from SK-N-AS cells. The 206-bp D2-DRE product was examined by gel electrophoresis/ethidium bromide staining. PCR analysis of the elution fractions in the absence (**lane 1**) or presence (**lane 2**) of anti-CC2D1A and its specific peptide control (**lane 3**; anti-CC2D1A antibody preincubated with 5-fold excess (mass/mass) of antigenic Freud-1 peptide). Controls included A7 genomic DNA (**lanes 5 and 6**), 1/10 of the input sheared chromatin (**lane 4**) and no DNA as a negative control (**lane 7**).

Regulation of endogenous dopamine-D2 receptor expression by Freud-1

To address whether Freud-1 regulates dopamine-D2 receptor expression, three human cell lines expressing different levels of dopamine-D2 receptors were examined (Arinami et al., 1997; Lin et al., 2001). The levels of DRD2 binding in A7, SK-N-AS and Y-79 cells were 6.7±1.5, 20.5±3.4, 35.3±4.4 fmol/mg protein, respectively. Two different antibodies detected the highest level of Freud-1 protein in the A7 cells (Figure III-6*A*), which expressed the lowest level of dopamine-D2 receptor number and mRNA, as analyzed by binding assays and QRT-PCR, respectively (Figure III-6*B*). Conversely, Y-79 cells displayed the highest level of dopamine-D2 receptor mRNA and binding and expressed the lowest level of Freud-1 protein. The inverse relationship between the level of Freud-1 and dopamine-D2 receptor expression is consistent with repressor activity of Freud-1 at the *DRD2* gene.

The role of Freud-1 in regulation of dopamine-D2 receptor expression was addressed using a specific Freud-1 siRNA to downregulate Freud-1 expression. The effectiveness of this siRNA was assessed in HEK293 cells. A marked depletion of Freud-1 protein was observed in cytosolic and nuclear fractions of Freud-1 siRNA-treated cells compared to non-transfected cells or scrambled siRNA control (Figure III-7*A*). The purity of fractions and equal loading was demonstrated by immunoreactivity of histone H1 (nuclear) and c-Raf (cytosol). Depletion of Freud-1 protein with Freud-1 siRNA also reduced the amount of protein/D2-DRE complex compared to scrambled siRNA control, as detected by EMSA (Figure III-7*B*, arrow), further substantiating the presence of Freud-1 in this complex.

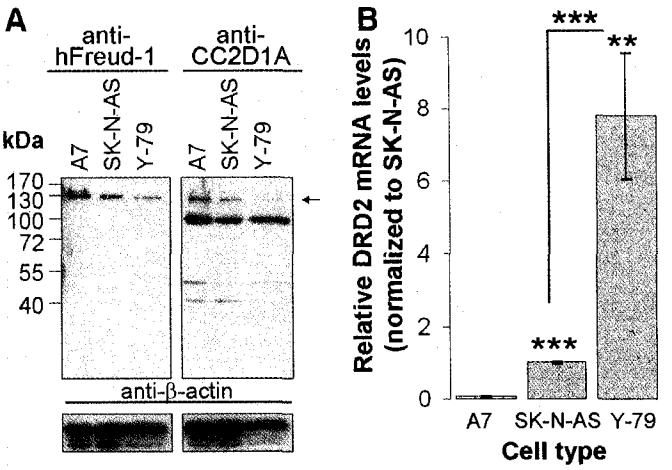

Figure III-6. Dopamine-D2 receptor mRNA expression is inversely related to Freud-1 expression level.

(**A**) Western blot analysis of Freud-1 protein level (**arrow**) in A7, SK-N-AS and Y-79 (50 µg) cells visualized with two specific human Freud-1 antibodies (**anti-hFreud-1** and **anti-CC2D1A**). Beta-actin was used as a loading control (**anti-β-actin**). (**B**) Quantification of dopamine-D2 receptor mRNA levels in A7, SK-N-AS and Y-79 cells with qPCR analysis using $2^{-\Delta\Delta C_T}$ method, with GAPDH RNA for normalization. The data are shown as dopamine-D2 RNA levels normalized to the level in SK-N-AS cells as average ±S.E. N≥3 where ***P≤0.0005 and **P≤0.005 (two-tailed unpaired t-test).

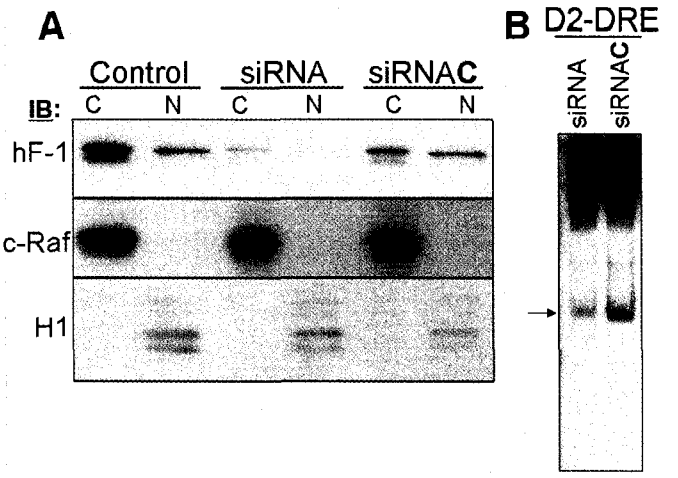

Figure III-7. Freud-1-specific siRNA reduced Freud-1 protein and protein/D2-DRE complexes.

(**A**) Down-regulation of nuclear (**N**) and cytosolic (**C**) Freud-1 by specific siRNA. HEK293 cells were untreated (**Control**), or treated with Freud-1 siRNA (**siRNA**) or scrambled control siRNA (**siRNAC**). Cell extracts were examined by Western blot analysis (25 μg/lane) using anti-hFreud-1 (**hF-1**), anti-c-Raf (cytosolic marker), and anti-histone-H1 antibodies (**H1**; nuclear marker). (**B**) Freud-1 siRNA reduces protein/D2-DRE complex (**arrow**) in HEK293 cells. Nuclear extracts from cells treated with either specific siRNA (**siRNA**) or scrambled control (**siRNAC**) were used in EMSA with ^{32}P-labeled D2-DRE as a probe.

The effect of Freud-1 siRNA on dopamine-D2 receptor mRNA expression was examined in D2-expressing cells since the *DRD2* gene is transcribed and can be regulated, unlike in HEK293 cells where the gene is completely silenced. Treatment with Freud-1 specific siRNA reduced Freud-1 protein level in all cell lines compared with non-transfected cells or cells transfected with scrambled siRNA (Figure III-8*A*). Conversely, Freud-1-specific siRNA but not scrambled siRNA, induced a significant increase in dopamine-D2 receptor mRNA and binding levels compared to untreated controls (Figure III-8*B*, *C*). The most pronounced increase in dopamine-D2 receptor RNA and binding levels was observed in A7 cells, which express high levels of Freud-1, but lower levels of dopamine-D2 receptors (Figure III-6). By contrast, siRNA weakly increased dopamine-D2 receptor mRNA in Y-79 cells which display the lowest levels of endogenous Freud-1 protein and the highest dopamine-D2 receptor levels (Figure III-6). Thus, the impact of Freud-1 siRNA on dopamine-D2 receptor levels was dependent on the level of endogenous Freud-1 expression. Therefore, Freud-1 represses the *DRD2* gene and regulates the basal level of DRD2 receptor expression in dopamine-D2 receptor-positive cells. Taken together, these results from reporter, EMSA and CHIP assays implicate the D2-DRE in negative regulation of *DRD2* gene expression by endogenous Freud-1.

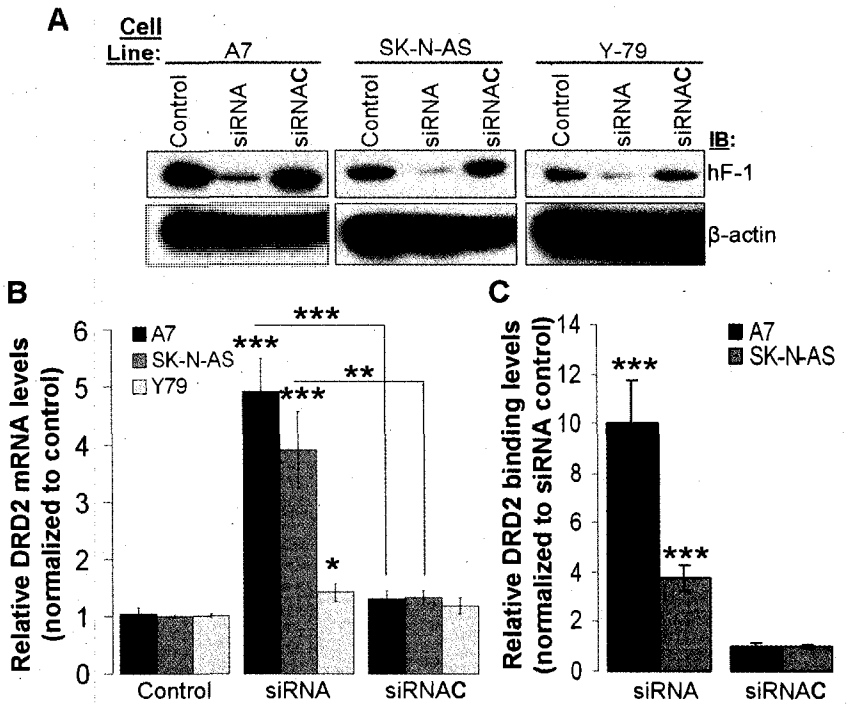

Figure III-8. Depletion of Freud-1 using siRNA increases dopamine-D2 mRNA and binding levels.

(**A**) Dopamine-D2-expressing A7, SK-N-AS or Y-79 cells were treated for 72 h with Freud-1-specific siRNA (**siRNA**) or scrambled siRNA control (**siRNAC**), and compared to non-transfected (**Control**) cells. Freud-1 protein was detected by Western blot analysis of whole cell lysates (50 μg/lane) using anti-hFreud-1 antibody (**hF-1**), followed by staining with β-actin as a loading control. (**B**) Up-regulation of dopamine-D2 receptor mRNA upon reduction of Freud-1 expression. QPCR analysis was used to quantify DRD2 mRNA levels in A7, SK-N-AS and Y-79 cells following treatment with Freud-1-specific siRNA (**siRNA**) or scrambled siRNA (**siRNAC**), compared to untreated control (**Control**). The data were analyzed using $2^{-\Delta\Delta C_T}$ method normalized to GAPDH mRNA and expressed relative to non-transfected cells and shown as average ±S.E. (N≥4). (**C**) Freud-1 specific siRNA upregulates DRD2 binding sites. Dopamine-D2 receptor binding

was quantified by specific [^{3}H]spiperone binding to cell membranes from A7 or SK-N-AS cells treated with Freud-1-specific siRNA normalized to specific control (**siRNAC**). Data represent mean±S.E. of three independent experiments. ***P≤0.0005, **P≤0.005, *P≤0.05 (two-tailed unpaired t-test).

3.5 DISCUSSION

Freud-1 mediated repression of the dopamine-D2 receptor gene

Dopamine is a key neurotransmitter that regulates a variety of physiological functions through the activation of dopamine receptors (Missale et al., 1998). Dys-regulation of dopamine-D2 receptor expression in humans has been implicated in schizophrenia, addiction and attention deficit hyperactivity disorder (ADHD) (Noble, 2000; Kapur and Seeman, 2001; Bobb et al., 2004). Transgenic animals overexpressing DRD2 in striatum display reduced working memory, implicating the level of dopamine-D2 receptor expression in cognitive development (Kellendonk et al., 2006). These findings indicate that the transcriptional regulation of the dopamine-D2 receptor gene may play an important role in these disorders; however, the transcriptional regulation of the human *DRD2* gene is poorly understood.

In this study, we have identified Freud-1 as a key transcriptional regulator of human *DRD2* gene expression at a conserved dual repressor element located in the second intron of the *DRD2* gene (D2-DRE). Freud-1 binds to this repressor element *in vitro* and in intact cells (Figure III-4 and Figure III-5). The D2-DRE mediated orientation-independent Freud-1 repressor activity, and point mutations shown to abrogate Freud-1 binding also impaired Freud-1-induced repression at the D2-DRE (Figure III-3). In addition to band-shift, supershift and reporter assays, several results demonstrate the crucial role of endogenous Freud-1 activity at the D2-DRE to regulate dopamine-D2 receptor expression. In particular, CHIP assays using Freud-1-specific antibody identified an interaction between endogenous Freud-1 and the second intron of the endogenous *DRD2* gene (Figure III-5). The interaction of Freud-1 with the D2-DRE

provides the first evidence of a transcription factor that binds to the human *DRD2* gene. Analysis of three different human cell lines revealed an inverse correlation between Freud-1 protein level and endogenous expression of dopamine-D2 receptor mRNA and protein levels (Figure III-6, Results), consistent with its role in basal repression of the DRD2 gene. In response to depletion of Freud-1 protein by siRNA treatment in these cell lines, expression of both DRD2 mRNA (Figure III-8*B*) and receptor levels (Fig 7*C*) were markedly up-regulated. Furthermore, both basal dopamine-D2 receptor expression and siRNA-induced up-regulation were proportional to the cellular content of Freud-1 protein (Figure III-6, 7*B*, 7*C*), indicating that Freud-1 is crucial for regulation of basal levels of human *DRD2* gene expression.

Very little is known regarding regulation of the human DRD2 promoter, which has been examined in only one study (Arinami et al., 1997) and has relatively weak activity compared with the rat DRD2 promoter. The rat *DRD2* gene has a robust TATA-less, CG-rich promoter that is driven by Sp1 factors and is typical of house-keeping genes (Minowa et al., 1992, 1994; Valdenaire et al., 1994; Yajima et al., 1997; Yajima et al., 1998; Hwang et al., 2001; Dunah et al., 2002). The nucleotide identity between rat and human DRD2 promoters is relatively low (58% over 500-bp vs. 74.6% for 5-HT1A promoter) and the human promoter lacks consensus sequences for several functional DNA elements (e.g., GATA, RARE, Sp1(B), AP2) (Minowa et al., 1992; Samad et al., 1997). Consequently, DNA elements located distal to the promoter may regulate the human *DRD2* gene as observed for other genes (Ogbourne and Antalis, 1998; Ainscough et al., 2000). Identification of the D2-DRE and its regulation by Freud-1 provides new insight into the importance of distal repressors in *DRD2* regulation. Although the role of

positive regulatory elements such as Sp1 in the human *DRD2* has not been studied, the finding that dopamine-D2 receptor expression is induced upon specific depletion of Freud-1 protein levels using Freud-1 siRNA indicates that Freud-1 is a key determinant of *DRD2* regulation in dopamine-D2 receptor positive cells.

Dopamine-D2 receptors in the brain are strongly expressed in post-synaptic regions such as cortex, striatum, nucleus accumbens, in addition to pre-synaptic dopaminergic neurons of the substantia nigra and ventral tegmental area (Mansour et al., 1990). Freud-1 is also expressed in pre-synaptic dopaminergic cells of the substantia nigra (Ou et al., 2003) and in embryonic and post-natal striatum and cortex, which express dopamine-D2 receptors (Basel-Vanagaite et al., 2006). The presence of Freud-1 in these dopamine-D2 receptor-expressing regions suggests that Freud-1 could play a role in regulation of pre- and post-synaptic dopamine-D2 receptor expression *in vivo*.

A functional polymorphism affects D2-DRE repression

In addition to identification of Freud-1 action to repress the *DRD2* gene, we also identified a novel functional polymorphism proximal to the D2-DRE that reduces Freud-1 binding and repressor activity. The A/G variation (rs2734836) is located 8-bp downstream of the D2-DRE and the G-allele attenuates Freud-1 binding and repressor activity (Figure III-3*B* and Figure III-4*D*). Nonetheless, D2-DRE G-allele retained repressor activity and weak binding of Freud-1. The frequency of the D2-DRE A-allele rs2734836 varies considerably depending on the ethnicity (0.042-0.5; NCBI) and is rare in Caucasians. The human cell lines used in this study had the GG genotype. Nevertheless, Freud-1 interacted with the genomic D2-DRE site (Figure III-5) and downregulation of Freud-1 derepressed dopamine-D2 receptor gene expression (Figure

III-8*B*, *C*) in these cells. Thus, although weaker than the A-allele, the D2-DRE G-allele retains significant Freud-1 binding and repressor activity.

Functional polymorphisms in DNA elements of candidate genes can have important effects on expression *in vivo*, perhaps accounting for predisposition to mental illnesses. For example, a functional 5-HT1A promoter polymorphism (C(-1019)G; rs6295), located at a different site from the 5-HT1A-DRE (located at -1519 (Ou et al., 2003)), has been associated with depression and suicide. The G(-1019) allele completely blocks the binding and repressor function of the transcription factor Deformed Epidermal Autoregulatory Factor 1 (DEAF-1) in the raphe nuclei (Lemonde et al., 2003). Interestingly, recent imaging studies associate the GG genotype of the C(-1019)G polymorphism with increased expression of 5-HT1A binding sites in the raphe region of medication-free depressed patients (Parsey et al., 2006a; Parsey et al., 2006b). Increased expression of 5-HT1A autoreceptors would reduce raphe firing, decreasing serotonin release as a possible mechanism for predisposition to depression and suicide. Since the A-allele of the D2-DRE displays enhanced Freud-1/D2-DRE interaction, a reduction in dopamine-D2 receptor expression is predicted. A decrease in pre-synaptic dopamine-D2 receptors would favour hyperactivity of dopamine neurons, a condition that is associated with enhanced reward, addiction and schizophrenia (Abi-Dargham et al., 2000; Noble, 2000; Seeman et al., 2005). On the other hand, reduced expression of post-synaptic dopamine-D2 receptors may reduce dopaminergic signalling. Future studies could address whether the D2-DRE polymorphism is associated with alterations in pre- or post-synaptic dopamine-D2 receptor expression *in vivo*, or is associated with mental illness.

Freud-1 function in vivo

These studies, together with previous findings, indicate that Freud-1 regulates both 5-HT1A and dopamine-D2 receptor gene expression. Although these receptors bear little sequence homology and their expression patterns are quite different, both are regulated by the same transcription factor, Freud-1. Interestingly, both receptors function as pre-synaptic autoreceptors to regulate serotonin and dopamine neurotransmission, respectively (Adell and Artigas, 2004; Albert and Lemonde, 2004). Thus, Freud-1 may coordinately regulate the activity of these two systems implicated in behavioural control.

The recent linkage of a deletion mutation in the *CC2D1A/Freud-1* gene with non-syndromic mental retardation (Basel-Vanagaite et al., 2006) and broad distribution of Freud-1 RNA and protein in the brain (Ou et al., 2003; Basel-Vanagaite et al., 2006), suggests its involvement in brain development and cognitive function. The deletion mutation of Freud-1 protein lacks domains essential for its repressor function (Ou et al., 2003), which likely results in a non-functional or dominant negative protein that could mediate upregulation of *DRD2* expression in receptor positive brain regions. Transgenic mice engineered to over-express dopamine-D2 receptors in striatum display impaired working memory (Kellendonk et al., 2006). This raises the interesting possibility that a reduction in Freud-1-mediated repression of the *DRD2* gene may contribute to the mental retardation phenotype or to other developmental disorders in which dys-regulation of the dopamine system is implicated, such as ADHD, autism or schizophrenia (Bobb et al., 2004). In some cases, mental retardation has been linked to global regulators of gene transcription, such as ATPase/helicase ATRX (alpha thalassemia/mental retardation syndrome X-linked) (Gibbons et al., 1995; Ausio et al., 2003) or methyl-binding repressor MeCP2 (methyl CpG binding protein 2) (Caballero and Hendrich, 2005; Fan

and Hutnick, 2005). Likewise, Freud-1 may regulate other genes in addition to 5-HT1A or dopamine-D2 receptor genes to regulate cognitive development. Further studies of the function of DRE-like elements in other genes may reveal additional gene targets for Freud-1 that could be implicated in cognitive development and mental retardation.

In summary, we have identified the human dopamine-D2 receptor as a new gene target for the repressor Freud-1. Our data implicate Freud-1/D2-DRE interactions in determining the level of dopamine-D2 receptors in D2-expressing cell types. Since Freud-1 is expressed in dopamine and serotonin neurons *in vivo*, as well as throughout development, Freud-1 regulation of 5-HT1A and dopamine-D2 receptor genes may coordinately regulate the maturation and function of serotonergic and dopaminergic systems.

CHAPTER IV - LINKAGE BETWEEN A NOVEL FUNCTIONAL DRD2 POLYMORPHISM AND THE TAQ1A VARIATION: ASSOCIATION STUDIES IN SCHIZOPHRENIA AND DEPRESSION DATASETS

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KEYWORDS: Transcription, repression, Freud-1, dopamine

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Authors' contributions: I have performed most of the work relating to the rs2734836 and rs2734835 SNPs as well as the data analysis. I have written the preliminary manuscript and Dr. Paul Albert and I have edited the follow up versions and have prepared it for submission. Ariel Burns has examined rs1800497 distribution in the data sets. Dr. Sylvie Lemonde has provided technical support and verified the results of the manual sequences. Dr. Evgeny I. Rogaev collected, analyzed and then generously shared his Russian Schizophrenic case-control group with our laboratory. Dr. Lisheng Du, David Bakish and Pavel D. Hrdina have collected MDD case-control group and generously provided it to our laboratory for analysis.

4.1 ABSTRACT

Altered regulation of the dopamine-D2 receptor gene (*DRD2)* has been implicated in mental illness and addiction, and genetic association studies have focused on the Taq1A polymorphism (rs1800497) in the *DRD2* locus. The minor T-allele of the Taq1A polymorphism has been associated with reduced DRD2 expression, but lacks a known function and appears to be linked to an unidentified functional polymorphism. We performed case-control association studies of age-, gender- and ethnicity-matched Russian schizophrenia and Canadian major depressive disorder datasets for Taq1A and novel *DRD2* polymorphisms (rs2734836, rs2734835). The rs2734836 and rs2734835 polymorphisms are located proximal to a *DRD2* repressor element that is regulated by the novel transcription factor Freud-1/CC2D1A. Importantly, the minor A-allele of rs2734836 is associated with decreased expression of dopamine-D2 receptors. No significant association was observed between the selected variations with either disorder, suggesting that these polymorphisms may not represent risk factors for schizophrenia or major depressive disorder in these cohorts. However, the possibility of a modest risk effect remains to be assessed in larger datasets. In both cohorts, the functional *DRD2* polymorphism (rs2734836) was in strong linkage disequilibrium with the Taq1A variation. In our samples, both minor alleles of these polymorphisms that associate with decreased DRD2 expression were covariant, indicating that the rs2734836 *DRD2* polymorphism is a functional polymorphism linked to the Taq1A polymorphism that can confer altered regulation of the *DRD2* gene. These data suggest that further studies of these polymorphisms in susceptible addiction or mental illness populations are warranted.

4.2 INTRODUCTION

Schizophrenia is correlated with increased activity of the mesolimbic and mesocortical dopamine systems (Abi-Dargham et al., 2000; Seeman and Kapur, 2000; Gainetdinov et al., 2001), and is treated with antipsychotic compounds that have the common property of antagonizing dopamine-D2 receptors (Missale et al., 1998; Noble, 2000; Lewis and Levitt, 2002). Binding studies in post-mortem brain tissue and imaging studies indicate that the dopamine-D2 receptor (DRD2) is upregulated in the basal forebrain of schizophrenics compared to controls, resulting in increased dopaminergic neurotransmission (Abi-Dargham et al., 2000; Seeman and Kapur, 2000; Gainetdinov et al., 2001). Altered regulation of the dopamine system has also been implicated in major depressive disorder (MDD), and some antidepressant treatments target dopamine-D2 receptors (Corrigan et al., 2000). A correlation between increased DRD2 levels and MDD has been observed (Klimek et al., 2002; Meyer et al., 2006), suggesting that alterations in transcriptional regulation of *DRD2* may contribute to the etiology of MDD.

We have previously identified a novel transcription factor, 5' Repressor Element Under Dual repression binding protein-1 or Coiled-coil and C2 Domain containing 1A (Freud-1/CC2D1A) that binds to a specific dual repressor DNA element to repress the serotonin-1A receptor (5-HT1A) gene (Ou et al., 2000; Ou et al., 2003). Importantly, a deletion in the Freud-1 gene was recently linked to autosomal recessive non-syndromic mental retardation (Basel-Vanagaite et al., 2003; Basel-Vanagaite et al., 2006), implicating Freud-1 in cognitive development and the regulation of additional genes. This is further supported by the widespread expression of Freud-1 in the brain region, including dopaminergic neurons in the substantia nigra (Ou et al., 2003). A genome-wide

scan revealed the presence of a conserved dual repressor element (D2-DRE) in the second intron of human *DRD2*, which is repressed by Freud-1 (Rogaeva et al., 2007a; Rogaeva et al.). Recently we investigated the rs2734836 polymorphism that is located 8-bp proximal to the D2-DRE, and demonstrated that the A-allele enhances Freud-1 binding and repression at the D2-DRE compared to the G-allele (Rogaeva et al., 2007b). These results indicate that rs2734836 is a novel functional polymorphism and that the allele-specific regulation of D2-DRE could alter dopamine-D2 receptor levels and predispose individuals to mental illnesses involving the dopamine system.

Previous studies of the *DRD2* locus have mainly focused on the Taq1A polymorphism (rs1800497), which is mapped adjacent to the *DRD2* 3'-untranslated region and is a non-synonymous variation within the ankyrin repeat and kinase domain containing 1 gene (*ANKK1*). The Taq1A polymorphism has been associated with schizophrenia (Golimbet et al., 2003), bipolar affective disorder (Li et al., 1999), alcoholism (Berggren et al., 2006), and Parkinson's disease (Grevle et al., 2000; Oliveri et al., 2000). However, these associations have not been consistently replicated (Noble, 2003). In addition, the Taq1A polymorphism has been associated with decreased levels of *DRD2* expression (Noble et al., 1991; Noble et al., 1997; Pohjalainen et al., 1998; Jonsson et al., 1999). However, the mechanism underlying this association is unclear. It has been suggested that an unknown polymorphism in the regulatory region of the *DRD2* locus is in linkage disequilibrium with the Taq1A polymorphism and is responsible for the reduced DRD2 levels and association trends (Pohjalainen et al., 1998; Jonsson et al., 1999; Neville et al., 2004).

In the current study, we investigated the Taq1A polymorphism and two previously uncharacterized *DRD2* polymorphisms located proximal to the D2-DRE (rs2734836 and rs2734835) in two case-control cohorts (Russian schizophrenia dataset and Canadian MDD dataset). No significant associations were identified in these cohorts. Importantly, the functional polymorphism (rs2734836) and the Taq1A variation were in strong linkage disequilibrium with each other in both cohorts. The A-allele of rs2734836 correlated with increased Freud-1-mediated repression of the *DRD2* was linked to the Taq1A T-allele associated with altered DRD2 receptor expression and mental illness (Noble et al., 1991; Jonsson et al., 1999; Golimbet et al., 2003; Rogaeva et al.), suggesting that rs2734836 is causally linked to the Taq1A associations in published datasets.

4.3 MATERIALS AND METHODS

Subjects

This study was approved by the Institution Review Boards of the Research Center of Mental Health (RCMH) of the Russian Academy of Medical Sciences (Russian schizophrenia case-control dataset (Chumakov et al., 2002; Goltsov et al., 2006)) and the Royal Ottawa Hospital (Canadian MDD case-control dataset (Lemonde et al., 2003)). Informed written consent was obtained from all subjects. The schizophrenia dataset included 151 patients diagnosed using both the International Classification of Disorders 10 and the DSM-III-R criteria for schizophrenia (Chumakov et al., 2002; Goltsov et al., 2006); and 160 normal control subjects (Table IV-I). The MDD dataset consisted of 166 Canadian (Caucasian) patients diagnosed using the DSM-IV criteria for major depressive disorder and 168 controls. All subjects in the MDD cohort had scores of 18 or greater on the 17-item Hamilton Rating Scale for Depression, which indicates moderate to severe depression. Control subjects in both datasets were matched to cases by age, sex and ethnicity.

Genotyping

Genomic DNA was isolated from blood samples as previously described (Lemonde et al., 2003; Golimbet et al., 2004). We genotyped three polymorphisms located in or nearby the *DRD2* gene. Two of the polymorphisms (rs2734835 and rs2734836), are located in the second intron of the *DRD2*. The third polymorphism, known as the Taq1A polymorphism (rs1800497), is located in the *ANKK1* and is at the 3' end of the *DRD2*. Genotypes for rs2734836 and rs2734835 were obtained by a direct sequencing approach. The PCR was done using two primers: 5'-ttccagggcagcttagtagagag-

Table IV-I. Characterization of the schizophrenia and major depressive disorder (MDD) case-control datasets.

N/A: not applicable.

Sample Characteristics	Schizophrenia Dataset		MDD dataset	
	Cases	Controls	Cases	Controls
Total Subjects	151	160	166	168
Mean age-at-onset (years)	21.4±6.8	N/A	43.1±8.7	N/A
Range age-at-onset (years)	6-49	N/A	23-69	N/A
Mean age of controls (years)	N/A	35.6±7.7	N/A	35.0±10.0
Range age of controls (years)	N/A	20-54	N/A	18-64
Female (%)	53	43	43	54
Ethnicity	Russian		Canadian Caucasian	

3', 5'-cccttctttcctacaaacacttatt-3' (Invitrogen, Burlington, ON, CA). The step-down PCR amplification cycles were: 92°C for 5 min; 92°C, 45 sec, 69°C, 45 sec; -0.5°C/cycle, 72°C for 90 sec (10 cycles); 92°C for 45 sec, 64°C for 45 sec, 72°C for 90 sec (30 cycles); and terminated at 72°C for 10 min. The PCR product was agarose gel-purified (GE HealthCare, Baie d'Urfe, QC, CA) and sequenced using 5'-tggagagtagttagggctg-3' primer (Invitrogen, Burlington, ON, CA) and the T7 sequencing kit (GE HealthCare, Baie d'Urfe, QC, CA) as previously described (Lemonde et al., 2003).

Genotyping of the Taq1A polymorphism was performed by TaqMan Assay (Applied Biosystems) using the Rotor-Gene 3000 Cycler (Corbett Research) with a thermocycler program: 95°C for 10 min; 40 cycles: 92°C for 15 sec, 60°C for 1min; hold at 25°C. The reaction was carried out in a 10µl volume containing: 1µl of DNA, 5µl of TaqMan Universal PCR Master Mix, 0.5µl of the 20X TaqMan Mix. The genotypes were determined using the Allelic Discrimination function of the Rotor-Gene software (version 6) and verified using 6 samples by PCR amplification of the SNP region followed by enzymatic digestion, using the protocol previously described (Grandy et al., 1993); genotypes obtained by the TaqMan assay and enzymatic digestion were concordant.

Statistical Analyses

All case-control analyses were computed using the software SNPAlyze Version 6.0.1 (statistical significance was taken to be $p<0.05$). Differences in the genotype or allele frequency between cases and controls were analyzed using the χ^2 test with Yeats' correction. The confidence interval was taken to be 95%. The genotypes for both datasets were examined independently for deviation from Hardy-Weinberg equilibrium. Benjamini corrected false discovery rate was used to correct for multiple testing

(Benjamini and Hochberg, 1995). Linkage disequilibrium (LD) between the SNP marker pairs was calculated using the SNPAlyze Version 6.0 software. The standardized, pair-wise Lewontin's disequilibrium coefficient (D') was employed to estimate the strength of LD between SNP markers. The effect of each genotype upon age-at-onset in the MDD and cohorts was evaluated by non-parametric analyses (Kruskal-Wallis test).

4.4 RESULTS

The genotypes for all three investigated polymorphisms (rs2734835, rs2734836 and Taq1A) were in Hardy-Weinberg equilibrium in the control samples from both the Schizophrenia and MDD datasets ($p \geq 0.197$). Sequencing of approximately 100 base pairs surrounding the D2-DRE in 482 individuals failed to detect additional polymorphisms in this regulatory region of the *DRD2*.

Statistical analysis of the MDD case-control dataset did not reveal any significant association between the disorder and the variations at the genotypic ($p \geq 0.34$) or allelic ($p \geq 0.27$) levels. Similarly, we did not detect a significant association between schizophrenia and any of the investigated polymorphisms (genotype $p \geq 0.26$; allele $p \geq 0.11$) (Table IV-II). Cumulatively these results suggest that the selected polymorphisms are not associated with MDD or schizophrenia in the investigated datasets. However, we detected a marginally significant modifying effect for rs2734835 on the age-of-onset in the patients with schizophrenia ($p=0.037$). The A-allele was predominant in individuals with late-onset schizophrenia where 52 individuals in schizophrenia cohort were affected after the age of 20 years, but after the Benjamini correction this association did not hold true.

We detected strong LD between all three examined polymorphisms in both cases and controls of the MDD and the schizophrenic case-control datasets ($p \leq 0.001$) (Table IV-III). The strong LD was observed between the Taq1A and rs2734836 polymorphisms ($0.90 \leq D' \geq 0.75$) despite the substantial distance between these two variations (~20.4-kb). The linkage disequilibrium coefficient for the two proximal polymorphisms (rs2734836 and rs2734835) ranged between 1 and 0.88.

Table IV-II. Association analysis of schizophrenia and major depressive disorder (MDD) datasets.

Shown are the data for the frequency of *DRD2* rs2734835, rs2734836 and the Taq1A (rs1800497; located in *ANKK1*) polymorphisms in the Russian schizophrenia and Canadian MDD case-control datasets. The genotype and allele frequencies are presented.

Dataset	rs#	Gene	SNP function	Chromosome position	Variation *major allele	#Cases/ #Controls	Genotype/ Allele	Frequency		Nominal p-value
								Cases	Controls	
Russian Scizophrenia Case-Control	rs2734835	*DRD2*	intron	112796553	A>C*	105/122	AA	0.21	0.20	0.413
							AC	0.49	0.42	
							CC	0.30	0.38	
							A	0.45	0.41	0.397
	rs2734836	*DRD2*	intron (functional)	112796449	A>G*	105/122	AA	0.02	0.02	0.878
							AG	0.26	0.25	
							GG	0.72	0.73	
							A	0.15	0.15	0.842
	rs1800497	*ANKK1*	coding nonsynonymous	112776038	C*>T	151/160	CC	0.64	0.71	0.262
							CT	0.32	0.27	
							TT	0.04	0.02	
							C	0.80	0.85	0.110
Canadian Major Depression Case-Control	rs2734835	*DRD2*	intron	112796553	A>C*	134/135	AA	0.11	0.17	0.338
							AC	0.53	0.47	
							CC	0.36	0.36	
							A	0.38	0.40	0.524
	rs2734836	*DRD2*	intron (functional)	112796449	A>G*	134/135	AA	0.03	0.02	0.654
							AG	0.30	0.28	
							GG	0.67	0.70	
							A	0.18	0.16	0.669
	rs1800497	*ANKK1*	coding-nonsynonymous	112776038	C*>T	155/129	CC	0.59	0.64	0.468
							CT	0.35	0.33	
							TT	0.06	0.03	
							C	0.77	0.81	0.266

Table IV-III. Linkage disequilibrium analysis.

Linkage disequilibrium between the *DRD2* rs2734835, rs2734836 (functional) and the Taq1A (rs1800497) polymorphisms were assessed using the Lewontin's disequilibrium coefficient (D') in schizophrenic and MDD case-control groups.

	Schizophrenic case-contol			Major Depression case-control		
Cases	**D'**					
SNP	**A/C (rs2734835)**	**A/G (rs2734836)**	**C/T (rs1800497)**	**A/C (rs2734835)**	**A/G (rs2734836)**	**C/T (rs1800497)**
A/C (rs2734835)		1	0.42		0.96	0.68
A/G (rs2734836)	0.22		0.75	0.33		0.91
C/T (rs1800497)	0.05	0.41		0.23	0.63	
	r^2					
Controls	**D'**					
SNP	**A/C (rs2734835)**	**A/G (rs2734836)**	**C/T (rs1800497)**	**A/C (rs2734835)**	**A/G (rs2734836)**	**C/T (rs1800497)**
A/C (rs2734835)		0.88	0.81		0.95	0.7
A/G (rs2734836)	0.19		0.9	0.24		0.88
C/T (rs1800497)	0.17	0.81		0.17	0.67	
	r^2					

4.5 DISCUSSION

The transcription factor Freud-1 is an important regulator of *DRD2* expression through the D2-DRE. Our prior study showed that one of the variations investigated here (rs2734836) is functional and affects the binding and repressor activity of Freud-1 (Rogaeva et al., 2007b). However, current results revealed that variations in *DRD2* flanking the repressor element (D2-DRE) do not constitute strong risk factors in our MDD and schizophrenia datasets. The lack of association with rs2734836 or the Taq1A polymorphism may be due to the limited sample size and the low frequency of the risk allele in our datasets (Table IV-II). Previously, the Taq1A polymorphism was more consistently associated with addictive disorders than with schizophrenia or MDD (Noble, 2003); therefore the association of these *DRD2* polymorphisms with addiction should be further investigated. Nevertheless, we detected a marginally significant modifying effect of rs2734835 on the age-of-onset in the patients with schizophrenia. The early-onset subgroup (before age 20) displayed association between the disease and the C-allele of rs2734835 ($p=0.037$). Indeed, early-onset schizophrenia is recognized to be a different sub-clinical category compared to the late-onset disorder (Lee et al., 2006). However, the function of rs2734835 has not been tested and biological significance of the observed association should be considered preliminary given the moderate sample size.

To our knowledge the current study provides the first evidence that a functional *DRD2* polymorphism, rs2734836, is in LD with the well-studied Taq1A variation. The A-allele of rs2734836 is known to be associated with increased binding and repression by Freud-1 leading to decreased DRD2 expression (Rogaeva et al., 2007b), similarly the T-allele of Taq1A is associated with reduced levels of DRD2 (Noble et al., 1991; Noble et

al., 1997; Pohjalainen et al., 1998; Jonsson et al., 1999). Our results revealed that the A-allele of rs2734836 is in strong LD with the T-allele of Taq1A (D' ranging from 0.75 to 0.90 in the investigated cohorts) (Table IV-III). This observation is in agreement with the haplotype-tagging variations in the *DRD2* locus identified from the HapMap project (http://www.hapmap.org). The CEU-population with ancestry from northern and western Europe revealed an extensive LD across the region investigated in current study. There are 35 variations in this region genotyped in the HapMap project, including two variations investigated in current study (rs2734836 and Taq1A). Both variations tag the same ~20.5kb haplotype consisting of at least 12 different variations (r^2 cut-off: 0.6).

Importantly, the Taq1A variation was reported to be associated with numerous disorders such as schizophrenia (Golimbet et al., 2003), bipolar affective disorder (Li et al., 1999), alcoholism (Berggren et al., 2006) and Parkinson's disease (Grevle et al., 2000; Oliveri et al., 2000). However, the functional role of the Taq1A polymorphism has never been demonstrated, suggesting that it may be in LD with one or more nearby functional polymorphisms. Taken together the LD between the Taq1A and rs2734836 polymorphisms and their association with decreased DRD2 expression could indicate that in fact rs2734836 is responsible for the changes in *DRD2* expression that was previously associated with the Taq1A variation. Future studies including the analysis of large datasets and additional variations in the *DRD2* region are essential to characterize the functional haplotype of the *DRD2* (Noble et al., 1991; Pohjalainen et al., 1998).

A recent study discovered that a deletion that affects the DNA-binding and repressor properties of *Freud-1* leads to a non-syndromic mental retardation suggesting a role for Freud-1 in cognitive development (Ou et al., 2003; Basel-Vanagaite et al., 2006;

Rogaeva et al., 2007a). It is possible that the rs2734836 A-allele may be responsible for the altered dopamine function associated with developmental disorders, such as autism or attention deficit hyperactivity disorder that are thought to involve dopaminergic hyperactivity. Future studies could address the association of the polymorphisms studied here with these cognitive disorders.

In summary, we identified the functional rs2734836 A-allele as a potential link accounting for the association of the Taq1A polymorphism with reduced *DRD2* expression, leading to the alteration in the dopamine system reported to be associated with different mental illnesses. Although our case-control study of three *DRD2* polymorphisms in the MDD and schizophrenia datasets did not reveal a significant association, further studies are warranted given the functional activity of the rs2734836 polymorphism and the limitations of the size of the cohorts in this study.

CHAPTER V - DISCUSSION

The main focus of this thesis has been on identifying and characterizing Freud-1 as a regulator of the *DRD2* and *HTR1A* expression. In the first manuscript we characterize a novel long isoform of Freud-1 (Freud-1_L) and its repressor activity at the previously characterized repressor element in the *HTR1A*. The Freud-1_L repressor activity has not been previously examined. Our data clearly demonstrates that the Freud-1_L has binding and repressor activity at the 5-HT1A DRE. We also demonstrate that overexpressing Freud-1_L further reduces promoter activity in the 5-HT1A DRE containing reporter constructs and endogenously Freud-1_L is bound to the DRE at the promoter of the *HTR1A*. Previously, our lab has identified Freud-1_S in the nuclear fraction (Ou et al., 2003) while initial examination of Freud-1_L localization has detected it primarily in the cytosol (Basel-Vanagaite et al., 2006). In this manuscript we address this discrepancy and try to identify a potential mechanism involved in regulating cellular distribution of Freud-1. Our results demonstrate that Freud-1_L is found in both nuclear and cytosolic fractions and that nuclear export is dependent on CRM1/exportin 1 protein.

In the second manuscript, we examined Freud-1 involvement in regulating transcription of the *DRD2*. Our data supports the hypothesis that Freud-1 is a negative regulator of the DRD2 at the conserved element (D2-DRE) located in the second intron. We present clear evidence that Freud-1 is bound to the D2-DRE both *in vitro* and endogenously. Furthermore, downregulating Freud-1 expression in cell lines endogenously expressing various levels of dopamine-D2 receptors reveals a negative regulation of the gene, since it results in increased DRD2 expression. In addition, examination of DRD2 and Freud-1 expression in the DRD2-positive cells reveals an

inverse correlation between their respective expressions. The analysis of the sequence surrounding the D2-DRE revealed two polymorphisms (rs2734835 and rs2734836) where the rs2734836 polymorphism was shown to be functional. Both the repressor activity and Freud-1 binding were affected by the rs2734836 polymorphism with the A-allele exhibiting stronger Freud-1 binding and repressor activity. These observations have lead to the final chapter of my thesis which has focused on examining the association of these polymorphisms with the two disorders affected by *DRD2* expression (Larisch et al., 1997; Pohjalainen et al., 1998; Jonsson et al., 1999; Abi-Dargham et al., 2000; Seeman and Kapur, 2000; Gainetdinov et al., 2001; Park et al., 2005).

In the final chapter we performed an association study of three polymorphisms with MDD and schizophrenia. The two previously uncharacterized polymorphisms, one of which alters D2-DRE activity as described in chapter III and a previously described and well studied SNP (Taq1A; rs1800497). Our selected sample group did not show an association with the selected disorders, but we did detect strong linkage disequilibrium between the functional polymorphism (rs2734836) and the Taq1A. Interestingly, Taq1A has been previously linked with a number of psychiatric disorders (Li et al., 1999; Grevle et al., 2000; Oliveri et al., 2000; Golimbet et al., 2003; Berggren et al., 2006) and low level of DRD2 expression (Noble et al., 1991; Noble et al., 1997; Pohjalainen et al., 1998; Jonsson et al., 1999). Therefore, the linkage disequilibrium between these polymorphisms provides evidence that the functional rs2734836 polymorphism would have also associated with the examined disorders (Li et al., 1999; Grevle et al., 2000; Oliveri et al., 2000; Golimbet et al., 2003; Berggren et al., 2006). Furthermore, this newly

identified polymorphism might be the missing link behind these positive associations and the decreased DRD2 levels (Figure V-9).

These three manuscripts provide significant contribution to the overall understanding of the transcriptional regulation of the *DRD2* and *HTR1A* genes implicated in mental disorders. We provide evidence that the Freud-1_L isoform is functional and regulates the *DRD2* at a differentially repressed element dependent on the allele of the proximal rs2734836 SNP. The significance of these findings is in the observation that the imbalance in the expression levels of the *DRD2* and *HTR1A*, which could be in part attributed to the altered transcriptional control, has been implicated in a number of mental disorders such as depression (Larisch et al., 1997; Li et al., 1999; Osterlund et al., 1999; Koks et al., 2006; Abbas et al., 2007), schizophrenia (Hashimoto et al., 1991; Hashimoto et al., 1993; Burnet et al., 1996; Arinami et al., 1997; Missale et al., 1998; Lewis and Levitt, 2002; Golimbet et al., 2003) and mental retardation (Berman and Noble, 1995; Sarnyai et al., 2000; Graybiel, 2005; Kellendonk et al., 2006). In addition, a recent finding that the deletion mutation in Freud-1 is linked to non-syndromic mental retardation (Basel-Vanagaite et al., 2003; Basel-Vanagaite et al., 2006) implicates Freud-1 function in cognitive development, potentially via its regulation of the *DRD2* or *HTR1A* expression. DRD2 overexpression reduces working memory (Kellendonk et al., 2006) and Parkinson's disease is associated with a decrease in short term working memory (Cooper et al., 1993). In addition, 5-HT1A KO mice demonstrate reduced cognitive ability (Sarnyai et al., 2000) while the 5-HT1A receptor antagonists enhance cognitive ability (Sumiyoshi and Meltzer, 2004; Schechter et al., 2005). This evidence implicates Freud-1 targets, such as the two genes described in this thesis, in cognitive function.

Despite the significant contribution there are a number of limitations to these studies. The major limitation concerns the functional analysis of the rs2734836 polymorphism. All the cell lines examined were homozygous for the G-allele; hence it was not possible to address the functionality of this polymorphism *in vivo*. Furthermore, we have analyzed the association of three polymorphisms with schizophrenia and MDD, and found no significant association within our cohorts, although there were limitations of ethnicity and sample size. It would be important in future studies to not only increase the sample size but to also examine additional ethnic groups for the association, especially given that the distribution of the functional polymorphism is quite variable depending on the ethnicity (NCBI database). Future research would have to focus on examining other disorders and sample groups for the association with the disease state. Furthermore, PET studies examining *DRD2* expression should be performed to assess an association between the genotype and the receptor levels.

5.1 ADDITIONAL FREUD-1 GENE TARGETS

Altered regulation of *HTR1A* and *DRD2* transcription may not entirely account for the role of Freud-1 in cognitive development that is suggested by linkage of the Freud-1 deletion to NSMR (Basel-Vanagaite et al., 2006). It is possible that additional gene targets of Freud-1 may participate in the mechanisms underlying the observed cognitive impairment in individuals with the deletion in *Freud-1*. One proposed factor is NF-κB, since transient transfection of Freud-1 induces transcription from an NF-κB-responsive element, suggesting that Freud-1 activates NF-κB (Matsuda et al., 2003). Mutation of Freud-1 may result in reduced NF-κB activity in affected individuals, perhaps contributing to cognitive dysfunction. Importantly, mice lacking either p50 or p65 subunits of NF-κB display reductions in learning or long-term memory in various tests, implicating NF-κB in cognitive function (Kassed et al., 2002; Meffert et al., 2003; O'Riordan et al., 2006). In the p50 knockout mouse, a reduction in anxiety has been observed (Kassed and Herkenham, 2004), suggesting that downregulation in Freud-1-induced NF-κB activity could result in decreased anxiety. Interestingly, NF-κB mediates mitogen-induced up-regulation of 5-HT1A receptor expression in human lymphocytes (Abdouh et al., 2001), and hence the NSMR mutation of Freud-1 might reduce expression of 5-HT1A receptors in the brain, perhaps mediating the effect of NF-κB on anxiety. To date the levels of 5-HT1A receptor expression have not been examined in these mice, but 5-HT1A KO have a well-characterized anxiety phenotype (Gross et al., 2002). Furthermore, *DRD2* is also regulated by NF-κB (Fiorentini et al., 2002; Bontempi et al., 2007) and the mutation in Freud-1 might abolish this inducible transcriptional control of *DRD2* expression at the NF-κB and the D2-DRE sites. As a result, mutation of Freud-1

would be expected to alter the overall regulation of transcription at both the positive and negative regulatory regions of *HTR1A*, *DRD2* and potentially other gene targets. Basel-Vanagaite *et al.*, with help of immunoprecipitation, did not detect a direct interaction between Freud-1 and p65 or p50 subunits of NF-κB *in vivo* (Basel-Vanagaite et al., 2006). Therefore, the effect of Freud-1 on the NF-κB is still uncharacterized and should be further addressed.

Additional targets of Freud-1 are of particular interest in order to understand the mechanisms by which Freud-1 regulates cognitive and emotional development. The BLAST search approach was used in this thesis to identify a functional D2-DRE with previously characterized DRE elements in the *HTR1A* (Ou et al., 2000; Ou et al., 2003; Lemonde et al., 2004). Therefore, additional targets might be identified in a similar manner; however, the consensus DRE sequence is quite degenerate and may result in multiple functional DREs being undetected due to lack of homology. On the other hand, identification of false positive, non-functional DREs would result in wasted time and resources. It is therefore essential to use a different approach to examine potential Freud-1 targets. One proposed alternative method is to perform CHIP assays from different cell lines then clone the immunoprecipitated DNA and sequence positive clones, or perform ChIP on Chip. The results might reveal DNA targets otherwise overlooked by other methods. In addition, advances in genomics provide a number of different techniques for the identification of transcription factor binding sites (Elnitski et al., 2006) as well as the gene arrays on the cells from healthy individuals verses ones carrying the mutation could identify genes affected by this mutation. Future work, however, would be needed to

identify which altered genes are directly affected by Freud-1 and by what mode of control.

5.2 FREUD-1 AND DISEASE

Previous data strongly implicates Freud-1 in negative regulation of 5-HT1A receptor expression. It is then reasonable to assume that the NSMR mutation, which is predicted to eliminate Freud-1 function as a repressor (Rogaeva et al., 2007a), could result in an upregulation of the *HTR1A* expression. Given that, 5-HT1A-overexpressing mice display reduced anxious phenotype (Kusserow et al., 2004), it is possible to assume that these individuals could have a less anxious state. Presently, there is no evidence on the anxiety or depression susceptibility of NSMR patients with Freud-1 deletion mutation; however, this would be difficult to examine in patients with MR. The relationship between Freud-1 and anxiety or depression is not yet established; however the 5-HT1A receptor is one of multiple factors that can contribute to depression (Albert and Lemonde, 2004; Berton and Nestler, 2006; Leonardo and Hen, 2006; Rogaeva et al., 2007a).

Increased *HTR1A* transcription in individuals with NSMR might contribute to cognitive impairment. Previous work has shown that 5-HT1A antagonists improve cognitive function (Harder and Ridley, 2000; Schechter et al., 2002; Schechter et al., 2005). Paradoxically, the 5-HT1A partial agonist (tandospirone) improves cognitive performance in medicated schizophrenics (Sumiyoshi et al., 2001a; Sumiyoshi et al., 2001b). Furthermore, a number of novel antipsychotics are 5-HT1A agonists and are thought to improve cognition (Newman-Tancredi et al., 2005; McCreary et al., 2007). Young 5-HT1A KO mice demonstrate impairment in cognition; however, this difference is attenuated with age (Wolff et al., 2004). Presently, it is unknown if the 5-HT1A

transgenic mice display altered cognitive function, although this would be important to assess (Rogaeva et al., 2007a).

Negative regulation of the *DRD2* by Freud-1 is particularly important given the implication of the dopamine-D2 receptor in memory and cognitive performance (Cooper et al., 1993). Animals overexpressing *DRD2* in the striatal region exhibit reduced working memory (Kellendonk et al., 2006). Furthermore, DRD2 agonists improve cognitive deficits (Ollat, 1992). Given the data presented in this thesis we suggest that a lack of functional Freud-1 in development might result in upregulated *DRD2* expression and consequently memory deficits. In addition, it is likely that other genes are regulated by Freud-1 and thus affected by the loss of Freud-1 function, contributing to the observed disease state.

These data necessitate verifying DNA-binding ability and functional activity of the mutated Freud-1. It would then provide a firm basis for the hypothesis that the developmental alterations in the 5-HT and DA systems due to altered transcriptional control of *HTR1A* and *DRD2* could account for the altered cognitive function resulting from the Freud-1 mutant found in NSMR carriers. This implication is important to examine in an animal model carrying a NSMR mutation in Freud-1 since it may be useful in evaluating the consequences on 5-HT1A and dopamine-D2 receptor expression, behaviour and cognitive function. In addition, individuals with NSMR should be examined for the levels of dopamine-D2 and 5-HT1A receptor expression and, if alterations in the expression are observed, potential drugs used to either enhance or reduce receptor activity might have beneficial properties to the disease state. Specifically, administration of these drugs at an early age using Freud-1 as a diagnostic marker could

be advantageous given that the signalling at the dopamine-D2 and 5-HT1A receptors is implicated in neuronal plasticity and neurogenesis (Banasr et al., 2004; Fricker et al., 2005; Kippin et al., 2005). Altering expression of some transcription factors such as Zif268 with antipsychotic drugs (haloperidol; DRD2 antagonists) (Nguyen et al., 1992) has been shown to upregulate *DRD2* (Bernard et al., 1991) expression and consequently might have an effect on the cognitive function. The same is true for 5-HT1A receptors, where agonist (8-OH-DPAT) stimulation reduced hippocampal phospho-CREB (Nishi and Azmitia, 1999) levels, which are known to be essential for synaptic plasticity and long-term memory (Kaltschmidt et al., 2006). In summary, the expression levels of Freud-1 targets are essential to examine in order to understand and design drugs for this complex disorder.

5.3 FREUD-1 MODIFICATION AND CO-FACTORS

The results of my thesis show that human Freud-1 is a repressor of both *HTR1A* and *DRD2*. However, the mechanisms by which Freud-1 mediates repression remain unclear. The regulation of the DRD2 and 5-HT1A promoters might be accompanied by recruitment of different cofactors. Previous analyses have shown that Freud-1 functions independently of HDAC (Lemonde et al., 2004), but it remains unclear whether other proteins might be involved in its repressor function. The sequence analysis of Freud-1 suggests that CtBP-1 might be a potential co-repressor that can achieve its function in an HDAC-dependent and -independent fashion (Chinnadurai, 2002). The human Freud-1_L includes three putative protein-protein interaction sites (consensus: P(V/L)DL(S/D); PVDLS, PDLS, PGDLD (Figure V-2)). Preliminary analysis using pull down assays has been unsuccessful at demonstrating this interaction (Figure V-3). Independent of this negative observation, the interaction with CtBP-1 might still take place under optimal conditions. The interaction might be transient or require Freud-1 modification such as phosphorylation. It is therefore valuable to mutate the putative CtBP-1 binding sites and examine the effect on repression by Freud-1.

Analysis of Freud-1 repressor function demonstrated its intrinsic repressor activity, but potential other proteins involved were not identified (Lemonde et al., 2004). In order to address whether other nuclear proteins interact with Freud-1, we have immunoprecipitated endogenous Freud-1 using anti-hFreud-1_L antibody, and identified the co-immunoprecipitated species by Mass Spectrometry analysis (Figure V-4). In these experiments we detected members of the chromatin remodelling SWI/SNF complex including BAF155 and BAF170. In preliminary experiments we successfully validated

this interaction through co-immunoprecipitation of Freud-1 with BAF155 and BAF170 (Figure V-5*A*). Therefore, we clearly linked Freud-1 repressor function with an ATPase-dependent chromatin remodelling SWI/SNF protein complex (Chi, 2004). The assessment of direct interaction between Freud-1_L and *in vitro* transcribed/translated BAF155, 170, 60a, BAF57 and Brg-1 was inconclusive (Figure V-6); however, we have identified calcium dependent interactions between Freud-1 and BAF155 (Figure V-5*B*). There are multiple explanations for the lack of interaction *in vitro*, one of which is absence of a protein that links Freud-1 with SWI/SNF complex in our examination assay. BAF57 has been reported to be this linker at times (Chen and Archer, 2005), but we did not observe direct interaction between BAF57 and the Freud-1 (Figure V-6). Another explanation for the lack of interaction is a requirement of Freud-1 to be phosphorylated or modified to form the interaction with its co-repressors. Thus, it is important to address this question and try to identify the linker between SWI/SNF and Freud-1.

Previous data from our laboratory has shown that ATP and Ca^{2+} inhibit Freud-1 binding to its repressor element. In addition, the activity of 5-HT1A promoter increased in the presence of elevated KCl levels while CaMK inhibitor reversed that effect (Ou et al., 2003). Taken together, these data suggest that CaMK attenuates Ca^{2+} dependent Freud-1-mediated repression. We have identified two putative CaMK phosphorylation sites (consensus: RXXS/T; RTFS and RRPT (Figure V-2)) (Yoshimura et al., 2003) and have shown it to be phosphorylated *in vitro* (Figure V-7). The direct interaction between Freud-1 and CaMK II and IV were not observed but an active form of kinase might still interact. Thus, it is important to address the contribution of Freud-1 phosphorylation by CaMKII/IV to its repressor activity and DNA binding.

Additional uncharacterized domains were also identified upon analysis of the human Freud-1_L sequence in the database (DM14, proline-rich region, coiled coils motifs; Figure I-17 and Figure V-2). The analysis also revealed one C2 domain of Freud-1 with high homology to Piccolo, a presynaptic cytomatrix protein found in the active zone where vesicles dock and fuse and therefore are involved in neurotransmitter release (Garcia et al., 2004). The Piccolo C2 domain is involved in homo- and hetero-dimerization thus the C2 domain of Freud-1 may also mediate protein-protein interactions. Presently this domain has been shown to be essential for Freud-1 DNA binding properties and repressor function (Ou et al., 2003), but additional implications of this protein domain are yet to be assessed.

It has been previously reported that post-translational modification of proteins affects their cellular distribution and function (Lin et al., 2003; Gee et al., 2006). The anti-hFreud-1_L antibody detects two specific bands differing in size by about 20-kDa. Cellular fractionation revealed the upper band to be more predominant in the nuclear fraction with the lower band primarily in the cytoplasm (Figure II-3). This observation has led to a hypothesis that the upper band is the Freud-1 protein modified by SUMOylation. This theory was based on the data indicating that SUMO-2/3 is about 13-kDa in size and previous research has shown 20-kDa difference in the migration of the protein consequent of SUMOylation (Sapetschnig et al., 2002) and its involvement in determining sub-cellular distribution of proteins (Fu et al., 2005). Freud-1_L sequence analysis identified three conserved SUMOylation sites (IKFE, FKTD and LKLD; Figure V-2) (Chung et al., 2004). Preliminary analysis revealed that immunoprecipitation using anti-hFreud-1_L and anti-SUMO-2/3 antibodies detected an immunoreactive band at the

same molecular size as Freud-1_L with anti-SUMO-2/3 antibody (Figure V-8*A*). On the other hand, immunoprecipitation with anti-SUMO-2/3 antibody and Western blotting with anti-hFreud-1_L antibody did not confirm this modification (Figure V-8*B*). There are number of explanation for this discrepancy, one being that the anti-SUMO-2/3 antibody is not able to immunoprecipitate SUMOylated Freud-1_L due to the protein tertiary structure. To further address this hypothesis a positive control should be used for the immunoprecipitation (e.g. c-Fos, (Bossis et al., 2005)). Alternatively, a different antibody should be chosen for this experiment. It is also possible that Freud-1 might be modified by a different method to achieve the observed 20-kDa supershift in its migration. Potential targets could be hyperphosphorylation as Freud-1_L contains many potential phosphorylation sites (Figure V-2) or poly-ADP ribosylation.

A number of these putative modifications and co-factor interactions could be essential for Freud-1 function. In addition, altered modification of Freud-1 could be one method to regulate its activity. It is important to further examine these possibilities in order to better understand Freud-1 activity at the repressor elements of the *HTR1A* and *DRD2 genes.*

5.4 CONCLUSIONS

Despite the limitations of these studies, this research has shed light on the transcriptional control of *DRD2* and *HTR1A* genes implicated in a number of disorders. Specifically, a new isoform of Freud-1 implicated in NSMR represses at a previously characterized repressor element in the *HTR1A*. We have also identified a novel target of Freud-1 repression, *DRD2*. The element in the *DRD2* was functional and bound Freud-1 endogenously. Furthermore, analysis of the sequence surrounding it revealed two previously uncharacterized polymorphisms, one of which had a functional effect on Freud-1 binding and the repressor activity (Figure V-9). The association analysis of these polymorphisms with schizophrenia and MDD did not reveal an association; however, one of them was found to be in strong linkage disequilibrium with a well characterized polymorphism. Therefore, this work provides the bases for a number of future projects and new interpretation of previously published work. The goal of this thesis was to understand the mechanisms behind transcriptional regulation; genes implicated and altered in disorders, as well as provide new ideas for therapeutic targets. In conclusion, our work provided significant contribution to the scientific community.

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APPENDICES

APPENDIX A

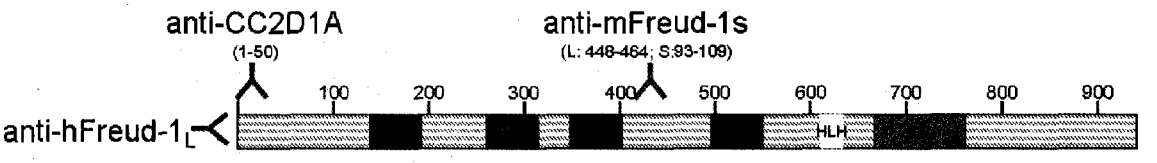

Figure V-1. Schematic representation of antigenic regions of Freud-1 specific antibodies. Three antibodies targeting Freud-1 (anti-hFreud-1_L, anti-CC2D1A and anti-mFreud-1_S) were used. The location of the recognition sequence is shown in brackets where the antigen for anti-mFreud-1_S antibody is located at position 448-464 in the long isoform (**L**) and 93-109 in the short isoform (**S**) of Freud-1. The antigen for anti-hFreud-1_L antibody is the full hFreud-1_L protein and for anti-CC2D1A are the first 50 amino acids at the N-terminal end.

APPENDIX B

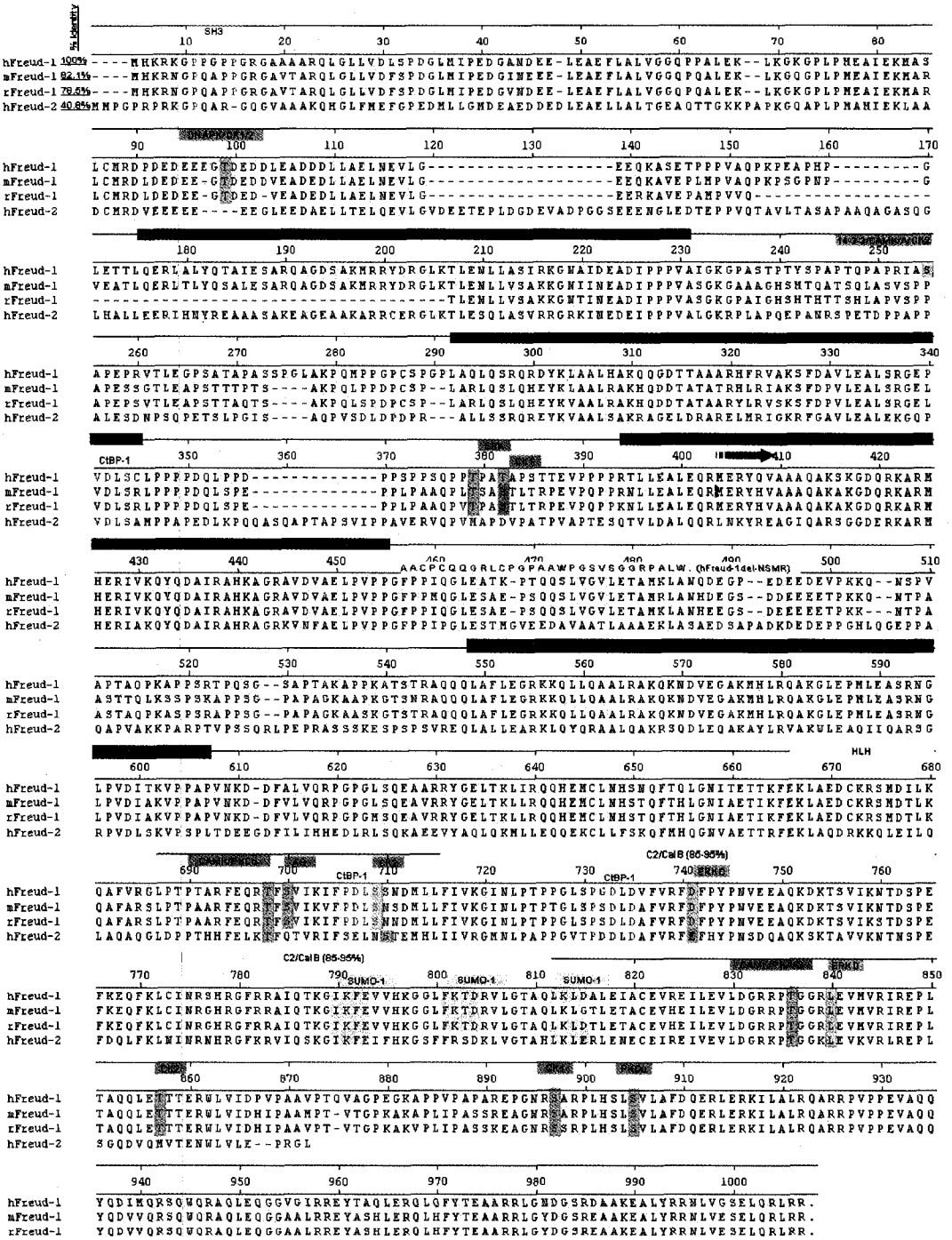

Figure V-2. Freud-1 alignment and putative domains.

Alignment of human (**h**), mouse (**m**) and rat (**r**) Freud-1 amino acid sequences with human Freud-2. Conserved domains are highlighted and % identity is given. Arrow

indicates the fist methionine in the mFreud-1_S, and the non-sense amino acid sequence found in NSMR patients is given above the deleted sequence. Yellow: interaction sites (SH3, C2, HLH); Green: interaction sites (ERKD, 14-3-3); Blue: kinase sites (CAMK, PKC, PKA, PKG, ERK, GSK3, DNAPK, CK1, CK2); Brown: modification sites (SUMO); Orange: CtBP-1 interaction sites.

APPENDIX C

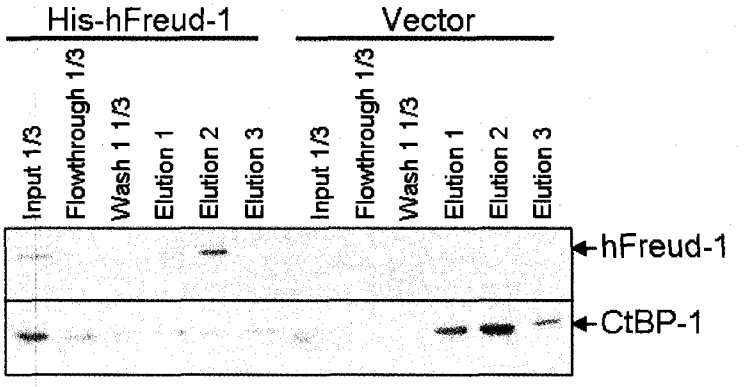

Figure V-3. Direct interaction between human Freud-1_L and CtBP-1.

Bacterially expressed His-tagged human Freud-1_L and CtBP-1 were preincubated and subjected to Ni-NTA pull-down assay to assess interaction between these proteins. Human Freud-1_L (**hFreud-1**) was successfully pulled out in the elution fractions 1 and 2 and CtBP-1 was also detected in those fractions; however, CtBP-1 was more abundant in the elution fraction in the absence of human Freud-1_L (vector negative control). Therefore, no specific interaction was detected between Freud-1_L and CtBP-1 as detected by anti-CtBP-1 and anti-S-tag antibodies.

APPENDIX D

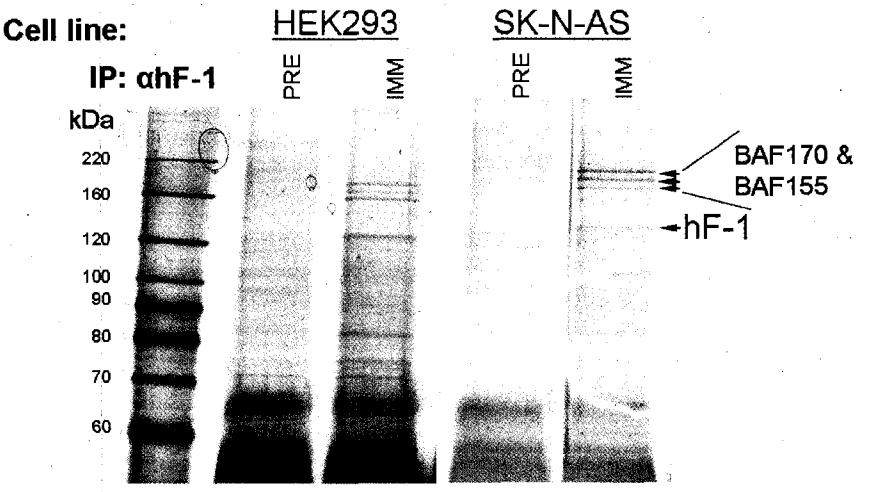

Figure V-4. Silver stain of immunoprecipitated endogenous human Freud-1 and its co-repressor complex.

Elution fractions of immunoprecipitated (**IP**) human Freud-1 (**hF-1**) with anti-hFreud-1_L antibody (**αhF-1**) from whole cell lysates of HEK293 and SK-NA-S cells. Preimmuned serum (**PRE**) was used as a negative control and compared with immunized (**IMM**) elution fraction. Arrows point to proteins identified by mass spectrometry as human Freud-1, BAF170 and BAF155.

APPENDIX E

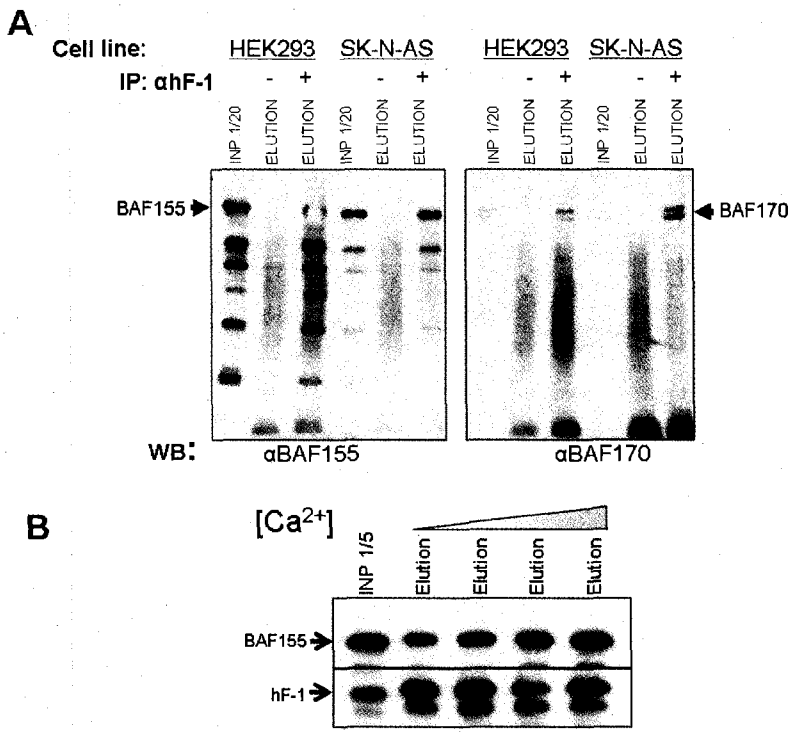

Figure V-5. Freud-1 interaction with BAF155 and 170 was enhanced by increasing Ca^{2+} concentration.

(**A**) Verification of the Mass Spectrometry results. Co-immunoprecipitation of human Freud-1 (**hF-1**) with anti-hFreud-1_L antibody (**αhF-1**) from HEK293 and SK-N-AS cells together with BAF155 and BAF170. Western blot analysis (**WB**) using anti-BAF155 (**αBAF155**) and anti-BAF170 (**αBAF170**) antibodies of the input (**INP**) and elution fractions following immunoprecipitation of human Freud-1 with αhF-1 (+) or preimmuned serum (-). (**B**) Co-immunoprecipitation of BAF155 with Freud-1 was enhanced by an increase in Ca^{2+} concentration. Input (**INP**) and elution fractions reveal co-immunoprecipitated BAF155 from HEK293 cells with αhF-1 in the presence of increasing Ca^{2+} concentration (0μM, 1μM, 10μM and 100μM). Western blotting for BAF155 reveals an increase in its enhanced co-immunoprecipitation in the presence of

higher Ca^{2+} concentration. Immunoprecipitation of Freud-1 was not affected by Ca^{2+} concentration as detected by αhF-1 (control).

APPENDIX F

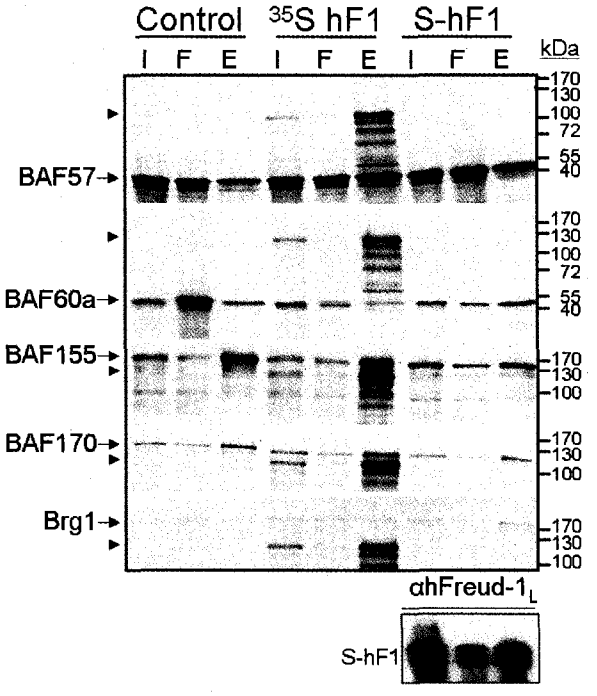

Figure V-6. Interaction between human Freud-1_L and examined components of SWI/SNF complex.

In vitro transcribed/translated human Freud-1_L (**^{35}S hF1**; **arrow head**) and bacterially expressed and purified S/His-tagged human Freud-1_L (**S-hF1**) was immunoprecipitated with anti-hFreud-1_L antibody. The successful immunoprecipitation of human Freud-1_L is demonstrated by the presence of either radioactively labelled human Freud-1_L or immunoreactivity with anti-hFreud-1_L antibody (**αhFreud-1_L**; last panel) in the elution (**E**) fraction. Input (**I**; 1/10), flowthrough (**F**; 1/10) and elution fractions were also loaded on the gel and analyzed for co-immunoprecipitation of ^{35}S-Met incorporated *in vitro* transcribed/translated BAF57, 60a, 155, 170 and Brg-1 (**arrow**). No obvious difference was detected between elution fractions of immunoprecipitation performed in the absence (presence of the empty vector; control) or presence of human Freud-1_L.

APPENDIX G

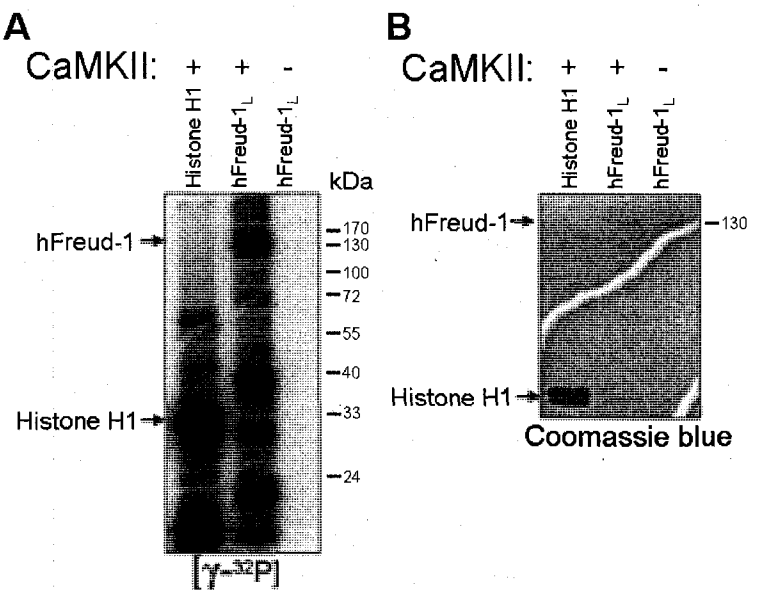

Figure V-7. *In vitro* phosphorylation of human Freud-1_L by CaMKII.

(**A**) Phosphorylation of human Freud-1_L. Bacterially expressed and purified His-tagged human Freud-1_L (**hFreud-1_L**; 1μg) is phosphorylated by CaMKII together with the positive control (**Histone H1**). Human Freud-1_L did not incorporate [γ-^{32}P] in the absence of the kinase (negative control). The kinase assay was performed as per New England Biolabs protocol. (**B**) Kinase assay loading control. Coomassie stain showing the presence of human Freud-1_L in both CaMKII containing (+) and CaMKII deficient (-) reactions.

APPENDIX H

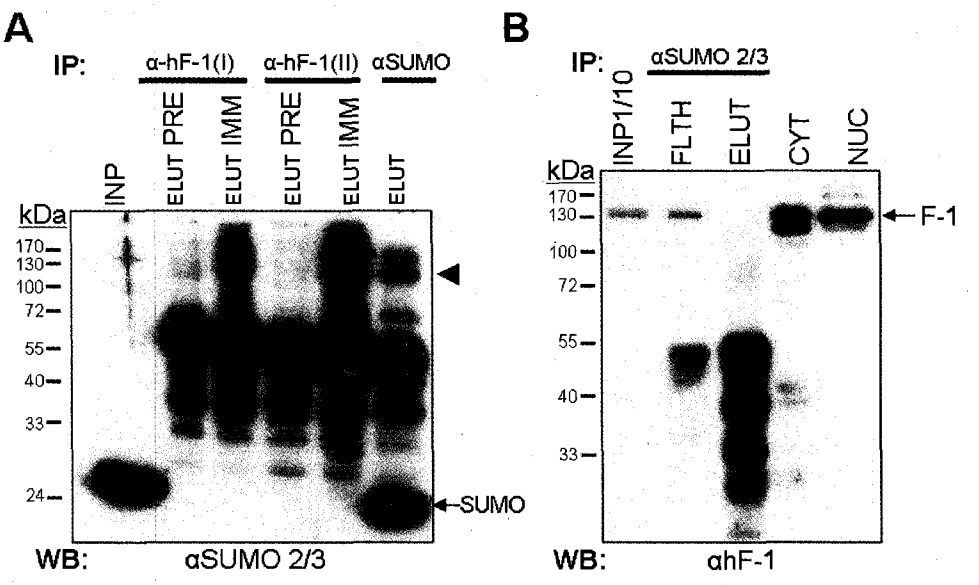

Figure V-8. SUMOylation of the endogenous human Freud-1_L.

(**A**) Immunoprecipitation of human Freud-1 and SUMOylated proteins from SK-N-AS cells. Two anti-hFreud-1_L (**α-hF-1(I)** and (**II**); **IMM**) and anti-SUMO 2/3 (**αSUMO**) antibodies were used together with anti-hFreud-1_L antibody specific negative controls (preimmuned serum; **PRE**). Western blotting with anti-SUMO 2/3 antibody (**αSUMO 2/3**) revealed high molecular weight SUMOylated proteins (**arrow head**) in the anti-hFreud-1_L antibody elution fractions (**ELUT**), contrary to the preimmune serum control. In addition, in the presence of the anti-SUMO 2/3 antibody, the elution fraction also immunoprecipitated a similar molecular sized proteins (**arrow head**) and SUMO 2/3 protein (**arrow**). (**B**) Immunoprecipitation of SUMOylated proteins from SK-N-AS cells using anti-SUMO 2/3 antibody (**αSUMO 2/3**). Freud-1 immunoprecipitation was analyzed by Western blotting with anti-hFreud-1_L antibody (**αhF-1**), revealing no human Freud-1 in the elution fraction (**ELUT**), contrary to input (**INP**) and flowthrough (**FLTH**). Cytosolic and nuclear proteins were loaded to illustrate the difference in the

migration of the two human Freud-1_L proteins in the nuclear (**NUC**) compared to cytosolic (**CYT**) fraction.

APPENDIX I

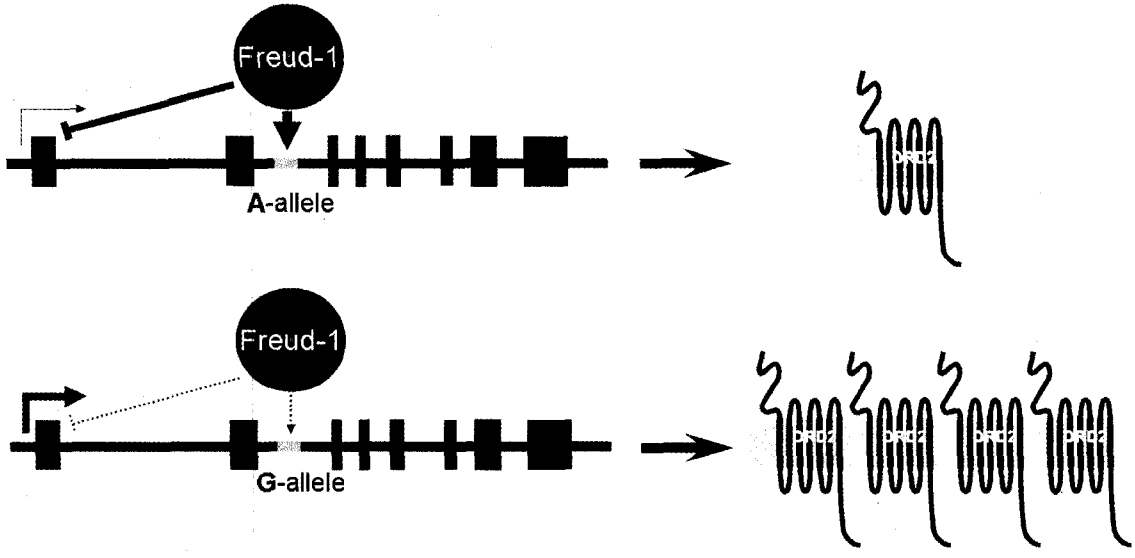

Figure V-9. Schematic representation of *DRD2* regulation by Freud-1.

Strong binding of Freud-1 to the A-allele (rs2734836) proximal to the repressor element reduces transcription of the *DRD2,* resulting in low receptor number. On the other hand, weak binding of Freud-1 to the G-allele (rs2734836) disinhibits transcription, resulting in upregulated DRD2 expression.